QUANTIFICATION OF FETOMATERNAL HAEMORRHAGE AND ITS APPLICATION IN ANTI-D DOSING IN RHESUS NEGATIVE MOTHERS DELIVERING AT MOI TEACHING AND REFERRAL HOSPITAL ELDORET.

BY

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A Thesis submitted to the School of Medicine in partial fulfillment of the requirements for an award of the degree of Master of Medicine in Reproductive Health

Moi University

DECLARATION

Student declaration

I declare that this research thesis is my original work and has not been presented in any other university or institution for the award of any degree or any academic credit.

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DEDICATION

To my family and friends for their constant support throughout my academic journey.

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ABBREVIATIONS

ACOG	American College of Obstetrics and Gynaecology
CS	Caesarian Section
FMH	Fetomaternal Hemorrhage
GOK	Government of Kenya
IREC	Institutional Research and Ethics Committee
ISO	International Organization for Standardization
KNH	Kenyatta National Hospital
MTRH	Moi Teaching and Referral Hospital
PGRH	Post-Graduate Reproductive Health
RH	Reproductive Health
RMBH	Riley Mother and Baby Hospital
SANAS	South African National Accreditation System
SOGC	Society of Obstetricians and Gynecologists of Canada
SOM	School of Medicine
SOP	Standard Operating Procedure
UNICEF	United Nations Children's Fund
WHO	World Health Organization

DEFINITION OF TERMS

Rhesus factor

Antigen found on the surface of red blood cells

Rhesus negative

Term denoting the absence of the Rhesus factor on the surface of red blood cells

Fetomaternal hemorrhage (FMH)

Entry of fetal cells into the maternal circulation during pregnancy or delivery

Large fetomaternal hemorrhage

FMH above 15ml of fetal whole blood but not exceeding 30ml

Excessive fetomaternal hemorrhage

FMH exceeding 30ml of fetal whole blood

Immunogenicity

Ability to induce a humoral or cell mediated immune response

Isoimmunisation

Production of antibodies against constituents of tissues of the same species

Kleihauer Betke test

Acid elution test used to detect and quantify fetomaternal hemorrhage

ABSTRACT

Background: Fetomaternal hemorrhage (FMH) is the entry of fetal blood into the maternal circulation during pregnancy or delivery. If this occurs in a Rhesus negative woman carrying a Rhesus positive fetus, there is a risk of the mother being sensitized against the D antigen. The effect is seen in the subsequent Rhesus positive pregnancies resulting in hemolytic disease of the fetus and newborn (HDFN). To prevent this sensitization, anti-D immunoglobulin is usually given. The dose given varies depending on the amount of FMH and protocols adopted by different professional authorities. There is limited local data on the determination of size of FMH and its utility in the dosing of prophylactic anti-D. The protocol in use in MTRH which is based on studies done in the West, recommends a uniform dose of 300µg irrespective of size of FMH, with no recommendation on quantification. Several studies have shown that lesser doses of anti-D can safely be used with lower cost implications.

Objectives: To determine the prevalence of Rhesus negativity, quantify the size of FMH and to determine the average calculated dose of anti-D immunoglobulin required for postpartum prophylaxis in Rhesus negative women delivering at MTRH.

Methods: This was a cross-sectional study conducted between April and September 2017. It involved estimation of size of fetomaternal hemorrhage, using the Kleihauer Betke test, on a sample of venous blood collected from Rhesus negative postpartum women within 2-12 hours after delivery. Consecutive sampling was used. Structured questionnaires were administered to eligible participants. Data analysis was done using R version 3.3.3 (R Core Team, 2017).

Results: Out of 4,552 deliveries over the study period of six months, 143 (3.1%) women were Rhesus negative. Of the 143 women, 99 met the eligibility criteria and were included in this study. The mean age was 26.4 years and mean gestational age at delivery was 39 weeks. Fetomaternal hemorrhage was detected in 35 (35.4 %) of the study participants, 24 (68.6%) of whom had FMH of less than 10ml. The size of FMH ranged from 2.5-20mls. The use of 100µg of anti-D immunoglobulin would have been sufficient for 89.9% (89/99) of the Rhesus negative mothers in whom quantification of FMH was done.

Conclusion: The prevalence of Rhesus negativity among deliveries in MTRH was 3.1%. FMH of less than 10ml occurred in 89.9% of study participants thus indicating that majority of the cases of FMH could have been neutralized by 100µg of anti-D immunoglobulin.

Recommendations: We recommend quantification of FMH in all unsensitized Rhesus negative women after delivery and accordingly adjusted dosing of anti-D immunoglobulin.

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CHAPTER ONE: INTRODUCTION

1.1 Background

The Rhesus blood group system is one of the most important human blood group systems. It consists of several antigens namely D, C, c and E with the D antigen being the most immunogenic. The presence of the antigen on the surface of red blood cells denotes one as Rhesus positive and its absence as Rhesus negative. (Landsteiner *et al,* 1940).

Being an antigen, the Rhesus factor can trigger an immune response with production of antibodies against it. This occurs when a Rhesus negative individual is exposed to Rhesus positive red blood cells (Landsteiner, 1941).

Unlike the ABO system where there are naturally occurring isoimmune anti-A and anti-B antibodies, Rhesus isoimmunization is a two-step process that involves sensitization followed by immunization. In sensitization, red cells carrying the Rhesus antigen sensitize immune competent cells to produce anti-D antibodies. The critical volume required for sensitization is about 0.1ml of blood. (ACOG Practice Bulletin No. 4, 2010).

Following subsequent exposure, the immune cells produce specific anti-D antibodies faster and more efficiently. The resultant effect is destruction of the Rhesus positive cells of the fetus by the maternal immune system via type II hypersensitivity reaction. The antibodies produced remain throughout life (Bowmann, 1989).

Entry of fetal red cells into maternal circulation during pregnancy or delivery is known as fetomaternal haemorrhage (Chown**,** 1954; Ahmed 2011). Potential obstetrical events that would result in fetomaternal haemorrhage include spontaneous or induced abortions, ectopic pregnancies, antepartum haemorrhage, external cephalic version, amniocentesis, cordocentesis, intrauterine fetal demise and abdominal trauma (Sebring, 1990).

If fetomaternal hemorrhage occurs in a Rhesus negative mother carrying a Rhesus positive fetus, the mother is at risk of being sensitized against the D antigen. The mother's immune system responds by producing antibodies against the D antigen present on the fetal red cells. The effect is seen in the subsequent D-positive pregnancies whereby fetal red cells are destroyed resulting in hemolytic disease of the fetus and newborn with its spectrum of erythroblastosis foetalis, icterus gravis neonatorum and congenital anemia of the newborn. To prevent sensitization from occurring Anti-D immunoglobulin is given.

The precise detection and measurement of fetal red blood cells in the maternal circulation is necessary for the prevention of Rhesus D isoimmunization among Rhesus negative women. The anti-D immunoglobulin dose required by a patient is calculated based on the size of fetomaternal hemorrhage. Several quantification methods exist and the two well established tests are flow cytometry and Kleihauer Betke test. The Kleihauer Betke test is an acid elution blood test used to measure the amount of fetal hemoglobin within the maternal circulation. It is the most widely used test as it is inexpensive, has a quick turnaround time and requires no specialist equipment (Al-Hassan, 2009).

Flow cytometry on the other hand works by using monoclonal antibodies directed against hemoglobin F. It has greater precision and accuracy compared to the Kleihauer Betke test, but a longer turnaround time and is more expensive.

1.2 Problem statement

The incidence of hemolytic disease of the fetus and newborn has been significantly reduced by the use of anti-D immunoglobulin prophylaxis from 13.2% to 0.2%. Assessing the amount of fetomaternal hemorrhage so as to give the right dose has further reduced the overall incidence to 0.14% (Kim Ya, 2012). This can further be reduced by strict compliance to guidelines concerning determination of fetomaternal hemorrhage and accordingly adjusted dosages of anti-D (JM Koelewjin, 2009).

The current practice in the country is that an unsensitized Rhesus negative mother who delivers a Rhesus positive child is given a dose of 300µg of anti-D immunoglobulin (National guidelines for quality obstetrics and perinatal care, 2011).The amount of fetomaternal hemorrhage varies among patients thus the common practice of a 300ug dose could be an under dose or overdose depending on the amount of fetomaternal hemorrhage that has taken place. The origin of the dose of 300µg is from a study done more than fifty years ago in North America with no clear indication as to why that particular dose was selected (Pollack, 1968).

In 1971, WHO suggested a standard dose of 200-300µg but stated that a 100µg dose was likely to have a success rate only slightly inferior to that of 200 µg. (WHO, Prevention of sensitization, 1971). Clinical experience in the UK has confirmed the

efficacy of the 100µg dose and this is the amount officially recommended for postpartum prophylaxis (Qureshi *et al,* 2014).

Similar studies done in Australia (Augustson *et al*, 2006) and Ethiopia (Urgessa *et al,* 2014) have shown that most cases of fetomaternal hemorrhage can be neutralized by lower doses than the current standard of 300µg.

Going by the Standard Operating Procedure (SOP) currently in use in MTRH, a Rhesus negative unsensitized mother receives a dose of 300µg at 28 weeks and another 300µg after delivery of Rhesus positive baby (MTRH, Department of Reproductive Health, Protocol no.33). As at now, measurement of fetomaternal hemorrhage is not part of the SOP. It is assumed that the 300µg of anti-D immunoglobulin given will cover any amount of FMH that has taken place despite the knowledge that the amounts vary based on patient and clinical factors (Sebring, 1990). No follow up is done on these patients and therefore we have no reports of under dosing or overdosing.

1.3 Justification

Rhesus isoimmunisation and disease remains a major public health problem in low and middle income countries (Zipursky, 2018) with causative factors such as scarcity of Anti-D, its prohibitive cost and lack of laboratory facilities for quantifying FMH implicated (Osaro, 2010). The is paucity of local data on determination of FMH and its utility in the dosing of Anti-D for postpartum prophylaxis, a study into this may have a significant contribution towards aiding decision making and improving maternal and neonatal health care.

A multicenter study in Australia established that anti-D dosing based on the size of fetomaternal hemorrhage instead of a standard dose for everyone would have resulted in an overall cost reduction of 30%. (Augustson *et al,* 2006). Being in a country where 46% of the population live under the poverty line (GOK-UNICEF Kenya Country Programme 2014-2018), quantification of fetomaternal hemorrhage and appropriate anti-D dosing may likely have lower cost implications.

1.4 Research questions

(i) What is the prevalence of Rhesus negativity among deliveries in MTRH?

(ii) What are the levels of fetomaternal hemorrhage in Rhesus negative mothers delivering in MTRH?

(iii) What is the average calculated dose of anti-D immunoglobulin required for postpartum prophylaxis in Rhesus negative mothers delivering in MTRH?

1.5 Objectives

1.5.1 Broad objective:

To calculate the amount of anti-D immunoglobulin required for post-partum prophylaxis in Rhesus negative women delivering in MTRH based on estimated volume of fetomaternal hemorrhage.

1.5.2 Specific objectives

1. To determine the prevalence of Rhesus negativity among women delivering in MTRH
2. To quantify fetomaternal hemorrhage in Rhesus negative deliveries
3. To calculate the amount of anti-D immunoglobulin required for post-partum prophylaxis in Rhesus negative mothers delivering in MTRH.

CHAPTER TWO: LITERATURE REVIEW

2.1 Rhesus phenotype

The Rhesus factor is an inherited protein found on the surface of red blood cells. It was discovered by Landsteiner and Weiner in 1939. It consists of several antigens namely D, C, c and E with the D antigen being more likely to initiate an immune response.

The incidence of Rhesus negativity varies by race and ethnicity as evidenced by studies done around the world.

In Africa, the frequency varies from country to country with Nigeria having a frequency of 4.4% (Jeremiah Z, 2005), Kenya 5% (Githiomi R, 2017), Guinea 4% (Loua et al, 2007) and Cameroon 2.4% (Tagny et al, 2009).

Meanwhile in the European and American region, the frequency of the Rhesus negative phenotype is significantly higher with the highest frequency being in the Basque populations at 30-35%, Caucasians in North America and Europe at 15% and African Americans at 8% (Zipursky A, 2011).

Towards the East, the frequency of Rhesus negativity is higher in India with a frequency of 5%. Farther East, the frequency is much lower with Japan having 0.5%, Thailand 0.3% and China 0.3% (Zipursky A, 2011).

The above mentioned studies however focused on general population. Published studies looking into the prevalence of Rhesus negative phenotype among pregnant women delivering in Kenyan facilities is limited. A literature search identified only one study done twenty years ago which found that the prevalence of Rhesus negative phenotype among pregnant patients attended to at the Aga Khan hospital was 3% (Mwangi J, 1999). Interracial marriages, especially with Caucasians from European countries

where Rhesus negativity is high, have increased by 85% since 1998. This could potentially alter the distribution and prevalence of the Rhesus negative phenotype.

2.2 Rhesus incompatibility in pregnancy

Rhesus factors are genetically determined. A baby can have the blood type and Rhesus factor of either parent or a combination of both parents. The Rh positive gene is dominant and will be expressed even in the heterozygous state (Mouru *et al,* 1993).

Therefore, if a Rhesus negative woman conceives with a Rhesus positive man that is homozygous for the Rhesus factor, the baby will be Rhesus positive. However, if she conceives with a Rhesus positive man that is heterozygous for the Rh factor, there is a 50% percent chance that the baby will be Rhesus positive (Willy Flagel, 2006).

2.3 Fetomaternal hemorrhage

Fetomaternal hemorrhage is defined as the entry of fetal red cells into maternal circulation during pregnancy or delivery (Chown*,* 1954; Ahmed, 2011).

Fetomaternal hemorrhage sufficient to cause isoimmunization occurs most commonly at birth in 15-50% of pregnancies (ACOG, Clinical guidelines Number 75, 2006). Abortions, ectopic pregnancies, antepartum hemorrhage, external cephalic version, in utero interventions, intrauterine fetal demise and abdominal trauma could also potentially result in FMH (Sebring, 1990; Osaro, 2010). The risk of isoimmunisation is directly proportional to the amount of fetomaternal hemorrhage that has taken place (Bowman, 1989).

Several studies have been done to assess the amount of fetomaternal hemorrhage in different populations. In one of the largest studies done on FMH, Auguston *et al* carried

out a 6 year multicenter trial on 5148 Rhesus negative mothers in Australia. He established that 98.5% of them had FMH of less than 2.5ml of fetal red cells and 90% had FMH of less than 1ml fetal red cells (Augustson *et al,* 2006).

In Ethiopia, a hospital based cross-sectional study on 75 Rhesus negative women detected FMH in 52% of the women with FMH of less than 10ml occurring in 92.5% of them. Only one patient (1.3%) was noted to have large FMH of more than 30ml (Urgessa *et al,* 2014).

In Germany, a prospective study in a teaching hospital demonstrated FMH in only 7.8% of patients with 94.2% of them having a FMH level of less than 10ml (David *et al,* 2004).

The amount of FMH has been shown to vary based on different patient and clinical factors such as age, gestation, mode of delivery, mode of placental delivery and presence of maternal medical conditions such as hypertension. (Sebring, 1990; Salim 2005; Kumari, 2017)

An extensive literature search did not identify any Kenyan studies focusing on quantification of fetomaternal hemorrhage. The knowledge of FMH among Rhesus negative women directs the correct Anti-D dose to administer in order to prevent Rhesus isoimmunisation.

2.4 Rhesus isoimmunisation during pregnancy

Rhesus isoimmunisation during pregnancy is as a result of fetomaternal hemorrhage occurring in a Rhesus negative mother carrying a Rhesus positive fetus (Chown, 1954).

Isoimmunisation is a two-step process that involves sensitization followed by immunisation. In sensitization, fetal red cells carrying the Rhesus antigen sensitize immune competent cells of the mother to produce anti-D antibodies. The critical volume required for sensitization is about 0.1ml of blood (ACOG Practice Bulletin No. 4, 2010). Following subsequent exposure, the immune cells produce specific anti-D antibodies faster and more efficiently.

The resultant effect is haemolysis of the fetal Rhesus positive cells by the maternal immune system. In mild forms this results in a mild fetal anemia but as the destruction of red blood cells worsen, hydrops fetalis develops and this can result in intrauterine fetal demise, premature deliveries and neonatal deaths.

In worst case scenarios, isoimmunisation may occur with resultant permanent biological damage manifested as recurrent pregnancy losses (Sisto Vechio *et al*, 2008).

In low and middle income countries, Rhesus isoimmunisation remains a major factor contributing to perinatal morbidity and mortality with estimates of causing 52,000 stillbirths, 98000 neonatal deaths and 17,000 cases of kernicterus annually (Zipursky, 2018). Factors that have been implicated to cause this include lack of knowledge by caregivers, lack of methods to assess FMH and the prohibitive high cost of Anti-D (Erhabor Osaro, 2010).

2.5 Methods of assessing fetomaternal hemorrhage

Kleihauer Betke test

The Kleihauer Betke test, discovered by Kleihauer, Braun and Betke in 1957, is an acid elution blood test used to measure the amount of fetal hemoglobin within the maternal circulation. It is a procedure employed in the management of Rhesus negative mothers

to determine the adequate dose of anti-D immunoglobulin required to prevent sensitization and immunization against Rhesus D positive cells of the fetus.

The test uses the principle that fetal red blood cells contain hemoglobin F that is resistant to changes in pH compared to adult hemoglobin. A sample of blood is taken from the mother's bloodstream at least within 72 hours of delivery or after a potential sensitizing event. A standard blood smear is prepared then subjected to an acid bath. The smear is then stained with a dye such as eosin and viewed under a light microscope. Fetal cells appear as darkly staining cells while maternal cells appear as ghost cells.

The fetal cells are expressed as a percentage of the total cells counted and then converted to total fetomaternal hemorrhage using one of the formulas below:

(i) Mollison's formula (1972): takes into consideration the following assumptions:

- *The maternal red cell volume is 1800 mL*
- *Fetal cells are 22% larger than maternal cells*
- *Only 92% of fetal cells stain darkly*

Therefore:

$$FMH\ (ml) = \frac{\text{No. of fetal cells per high power field} \quad X \quad 1800 \quad X \quad 122 \quad X \quad 100}{\text{No. of maternal cells per high power field} \qquad 100 \qquad 92}$$

(ii) American Association of Blood Banks formula (AABB) (2008):

FMH (ml) = (% of fetal cells by Kleihauer Betke/100) x 5000

The disadvantage of the Mollisons formula is that it takes a lot of assumptions into consideration and is laborious to compute compared to the AABB formula. However, the results yielded using either formula are not drastically different with Mollison's

formula yielding a slightly lower amount of FMH by about 0.8ml of whole blood (Kim, 2012). The AABB formula was used in this study.

The advantages of the Kleihauer Betke test are that, in addition to detecting fetal bleeds as small as 2.5ml, it is also inexpensive, requires no special equipment and has a quick turn-around time (Al-Hassan *et al*, 2009). The cost in Kenya is approximately Ksh.800 (Pathologists Lancet Price list catalogue, 2015). This allows it to be performed at low resource centers. A systematic review done on its sensitivity concluded that the sensitivity of the test ranges between 70-92% (Eshel *et al,* 2018). The main disadvantage of the KHB test is that it is unable to distinguish fetal cells from maternal cells containing hemoglobin F (Weaver *et al*, 1990). Maternal cells with hemoglobin F would also be counted as fetal cells and therefore give a false positive result or an overestimation on the magnitude of FMH (Stropnicky *et al*, 1982). It is for this reason that its use in patients with hemoglobinopathies such as sickle cell disease is discouraged (Krauss *et al*, 1983).

Flow cytometry

The flow cytometry method of assessing fetomaternal hemorrhage is based on the detection of minor population of D positive cells with a fluorochrome conjugated IgG monoclonal anti-D reagent. A monoclonal antibody to hemoglobin F is conjugated to a fluorochrome and used to detect fetal hemoglobin in permeabilized cells as they pass through the channel of a flow cytometer (BCSH FMH guidelines, 2009).

Flow cytometry has been shown to be less labour intensive, have less inter-observer variability and the added advantage of differentiating between fetal red cells and adult cells containing hemoglobin F. It is, however, more expensive with a long turn-around time of 72 hours (Bromilow *et al*, 1997 & Al-Hassan *et al*, 2009). The cost of flow

cytometry in Kenya is approximately Ksh.3000 (Lancet Price list catalogue, 2018). Its sensitivity in detecting and quantifying FMH is comparable to the Kleihauer Betke test (Eshel *et al*, 2018).

Rosette test

The rosette test utilises an indirect antiglobulin test with increased sensitivity achieved by the addition of Rh D positive "indicator" red cells. These indicator cells adhere to the anti-D coating the fetal red cells that have the Rhesus antigen. This results in clusters that can be visualized under microscopy and counted. This method is able to identify bleeds as little as 2.5ml (Australian and New Zealand Society of blood transfusion, 2002). However, this is more of a screening test necessitating other methods to quantify the FMH.

Gel agglutination test

This test utilizes the principle of antibody-antigen reactions. Serial dilutions of monoclonal IgG anti-D are incubated with the test sample at 37ºC for an hour. The cells are then centrifuged and tested against a similar amount of Rhesus positive red blood cells using gel cards. The agglutination reactions are then graded as positive or negative based on the presence and degree of agglutination (BCSH FMH guidelines, 2009).

2.6 Anti-D Immunoglobulin

Anti-D is a solution of IgG anti-D that is produced by the pooling and fractionating of plasma from a large number of Rhesus negative men that have been exposed to Rhesus positive red blood cells to stimulate the production of Rhesus antibodies (Finn, 1961).

It is administered to Rhesus negative mothers carrying Rhesus positive babies to neutralise any fetal Rhesus positive red blood cells that entered the maternal circulation during fetomaternal hemorrhage. This prevents the maternal immune system from

initiating a cascade of events that would lead to sensitization (Finn *et al*, 1961). It is sold under different trade names such as Rhogam, MicRhogam, KamRho, Rhoclone etc and in formulations of 300µg and 100µg. The dose of 300µg retails at Ksh.5000 and that of 100µg at Ksh.3500 (KEMSA pricelist, 2018).

The timing and amount given vary as different countries have different protocols. A Cochrane review on Anti-D administration after childbirth to prevent Rhesus alloimmunisation concluded that while its use is effective, the data on the optimal amount for prophylaxis is limited. The review noted that the recommendations on dose in different countries depend on availability and cost of anti-D immunoglobulin as well as the costs of laboratory assessment of fetomaternal hemorrhage (Crowther, 2013).

According to the ACOG guidelines, it is recommended that anti-D immunoglobulin be given at 28 weeks gestation, postpartum and in the event of obstetrical sensitizing events at a dose of 300µg (ACOG Practice Bulletin No. 4, 2010). This is similar to the guidelines in Canada as directed by the Society of Obstetricians and Gynecologists of Canada (Fung *et al*, 2018) as well as that in use in MTRH.

The origin of the 300µg dose can be traced back to a study done fifty-six years ago that looked into the results of clinical trials on the use of Anti-D. The study compared a dose of 5000-6000µg of Anti-D versus 300µg. The dose of 5000-6000µg is the dose that was initially used for the prevention of Rhesus isoimmunisation since the advent of Anti-D (Finn *et al,* 1961). The findings showed that the latter dose conferred protection similar to the high dose (Pollack *et al*, 1968). However, the reasons as to why a dose of 300µg was chosen in that study is not clear.

Ideally, the dose of anti-D immunoglobulin should be determined according to the level of exposure to Rhesus positive red blood cells and based on the knowledge that 0.5 ml

of packed Rhesus positive red blood cells or 1 ml of Rhesus positive blood is neutralised by approximately 10µg of anti-D (Crowther, 2000).

As earlier stated, the amount of FMH varies with patient and clinical factors (Sebring, 1990). In Australia and New Zealand, they are cognisant of this and therefore recommend that the appropriate dose of anti-D should be calculated based on the volume of FMH (RANZCOG guidelines, 2018). Studies in the UK showed that most cases of FMH in their set up can be neutralized by 100µg of Anti-D and thus recommend that dose for postpartum prophylaxis (Qureshi, 2014).

Kenyan studies on Rhesus negative blood group mainly focus on prevalence in the general population and management of those already isoimmunised. Only one study looking into FMH was identified. This study however did not quantify the levels, rather it looked at whether FMH occurred or not and recommended further studies into determining the right dose of anti-D required in our set-up (Mulandi, 1984). A look into the Kenyan national guidelines is unclear on what directed the dose of 300µg for postpartum prophylaxis (National guidelines for quality obstetrics and perinatal care, 2011). National clinical management and referral guidelines contradict the dose of 300µg and recommend 500µg instead for postpartum prophylaxis (MOH, clinical management and referral guidelines, volume III). The scientific basis for either dose in our set-up is lacking and therefore it is difficult to conclude on whether 300µg is an under dose, overdose or the right dose for Rhesus negative women delivering in Kenyan facilities.

Under dosing carries an increased risk of isoimmuisation whose consequences include recurrent pregnancy losses, increased perinatal morbidity and mortality and increased

costs in management of affected individuals (Erhabor Osaro, 2010; Sisto Vechio, 2008).

Overdosing increases the risk of adverse events that a patient may experience. Adverse effects ranging from mild to severe have been reported. Mild adverse effects include nausea, generalized chills, joint pains, urticaria and pain at the injection site. Severe effects occur to a lesser extent and include disseminated intravascular coagulation, anaphylaxis, hemolytic uremic syndrome, myocardial infarction, adult respiratory distress syndrome and renal failure (Bussel *et al*, 1991; CSL Behring, 2012).

Adequate use of anti-D has been shown to significantly reduce the risk of sensitization from 16% to 1.9% (Bowmann *et al*, 1978) and reduce the incidence of hemolytic disease of the fetus and newborn from 13.2% to 0.2% (Kim Ya, 2012) as well as having lower cost implications of as much as 30% (Augustson *et al*,2006).

CHAPTER THREE: METHODS

3.1 Study design

This was a cross sectional study. It consisted of quantifying the amount of fetomaternal hemorrhage that takes place in Rhesus negative mothers delivering at RMBH as well as establishing the total number of rhesus negative mothers that deliver in MTRH.

3.2 Study area

The study was conducted at the Riley Mother and Baby Hospital (RMBH) wing of the Moi Teaching and Referral Hospital. This is the second largest referral hospital in Kenya catering to a population of about 15 million. Riley Mother and Baby Hospital is a well-established obstetric unit offering obstetric services to mothers in the Western region of Kenya. It handles approximately 900 deliveries every month (Moi Teaching and Referral Hospital records, 2015).

At the time of the study, the MTRH labs did not have the capacity to do the Kleihauer Betke test thus Lancet laboratories were used. Lancet is one of the leading pathology laboratory services in Africa and is a South African National Accreditation System (SANAS) accredited laboratory that adheres to internationally set criteria according to ISO 15189.

3.3 Study population and target population

The study population were women delivering at RMBH of MTRH. The target population were Rhesus negative mothers meeting the inclusion criteria.

3.4 Sample size

The main objective of the study was to quantify fetomaternal hemorrhage and calculate the dose of postpartum Anti-D immunoglobulin prophylaxis required by Rhesus negative women delivering in Riley Mother Baby Hospital of MTRH.

We determined the number of Rhesus negative participants required in order to sufficiently answer the objective of the study with 95% confidence using the following formula (Cochran, 1963),

$$n = \left(\frac{Z_{1-\alpha/2}}{d}\right)^2 \times P(1-P)$$

$$= \left(\frac{1.96}{0.03}\right)^2 \times 0.9 \times (1-0.9)$$

$$= 385$$

Where:

P = 90.0% is the proportion of participants with FMH <0.1 ml among the Rhesus negative mothers (Solomonia et al, 2012).

d = 0.03 is the margin of error. We used a small margin of error (3.0%) in order to ensure we get a sufficiently bigger sample size to avoid sampling participants with FMH<0.1 ml only.

$Z_{1-\alpha/2}$ is the quartile of the standard normal distribution corresponding to $(1-\alpha/2)\times 100\%$ percentile.

Due to the low prevalence of Rhesus negative blood group, correction was done for finite population. Preliminary data from MTRH showed that 2.8% of the mothers delivering at RMBH are Rhesus negative (MTRH Reproductive Health records, February 2016). On average 900 mothers deliver in MTRH per month. This gave approximately 25 Rhesus negative mothers per month and 150 in six months.

The formula below was then used to adjust for finite population:

$$\frac{n}{1+n/N} = \frac{385}{1+385/150} = 92$$

Factoring in a laboratory rejection rate of 10%, the formula below was employed:

$$\frac{n}{1 - W}$$ where n = 92 and W = 0.1

This yielded a sample size of 103. During data collection, no sample had been rejected by the time 99 study participants had been recruited and therefore collection was stopped.

3.5 Sampling method

Consecutive sampling technique was used in this study to pick the mothers. This is mainly due to the fact that Rhesus negative individuals are relatively few (Mwangi J, 1999 and Mulandi TN, 1985). Rhesus negative mothers who were admitted to the labour ward had the indirect coombs test done as per hospital protocol to determine whether they were isoimmunised or not. Those who were not isoimmunised and met the inclusion criteria were approached after delivery by either the principal investigator or research assistants and informed about the nature and purpose of the study as well as its potential risks and benefits. Those who gave informed consent were then recruited into the study. The study participants were then interviewed and 2mls of venous blood taken for FMH estimation within 2 –12 hours after delivery.

3.6 Eligibility criteria

3.6.1 Inclusion criteria:

Rhesus negative unsensitized mothers as they do not benefit from the use of Anti-D.

Deliveries after 28 weeks gestation. The occurrence of FMH in those below 28 weeks is only 1.9% (Kumari, 2017).

3.6.2 Exclusion criteria:

Rhesus negative mothers that had already received Anti-D after the current delivery.

Patients who are known to have sickle cell disease. This is due to the elevated levels of haemoglobin F containing red cells seen in patients with the disease. These cells could

mistakenly be identified as fetal cells by the Kleihauer Betke test and therefore give falsely high levels of FMH.

STUDY PROCEDURE

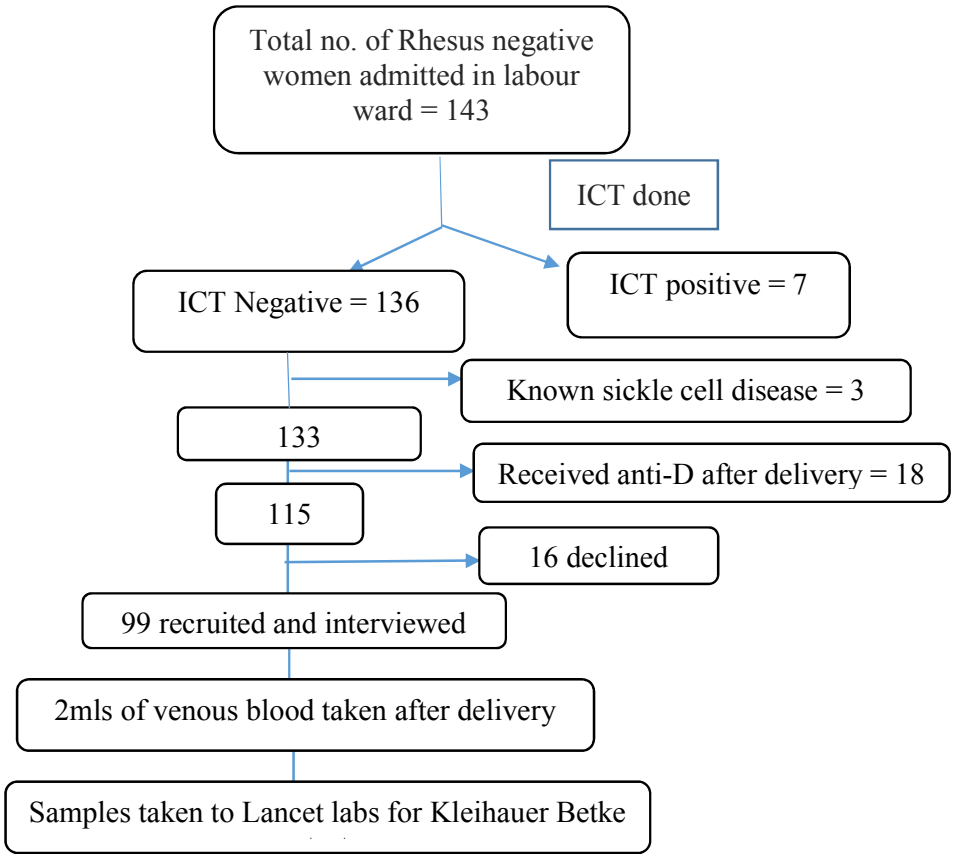

Figure 1: Study procedure

3.7 Data collection

Data was collected by the principal investigator and two research assistants. The research assistants, both of whom were nurses, were trained on how to administer the questionnaire, optimize sample collection, labelling and storage. Data was collected from April 2017 to September 2017. Data on the number of deliveries as well as number of Rhesus negative mothers delivering at the facility during the study period was collected from MTRH Reproductive Health records. The number of Rhesus negative mothers delivering at the hospital was recorded daily. This information was used to calculate the prevalence of Rhesus negative mothers delivering at the hospital.

Sociodemographic, medical and obstetric characteristics of the study participants were collected using an interviewer-administered structured questionnaire. Medical records were reviewed and relevant clinical data was abstracted and entered into the structured questionnaire.

3.8 Laboratory analysis of FMH

A preliminary visit was made to the Lancet laboratories. This was to work out feasibility, logistics and procedures concerning the Kleihauer Betke test.

For FMH estimation, 2ml of venous blood from each study participant was collected in an EDTA bottle between 2-12 hours after delivery. This ensured sufficient time to elapse to allow fetal cells to be distributed within the maternal circulation after delivery. The samples were then stored in a refrigerator in the labour ward at a temperature of 4°C and transported in a cooler box to the Lancet laboratory for further processing.

At the laboratory, thin blood slides were made, air dried and fixed with 80% ethanol. The citrate phosphate buffer (pH 3.3) used for elution was warmed to 37°C and prepared slides immersed in it for 5 minutes. The slides were then thoroughly washed with distilled water and air dried completely. Thereafter, staining was done for 3 minutes with acid hematoxylin solution after which the slides were rinsed with distilled water and counterstained with 0.1% eosin B solution for 4 minutes.

A dry coverslip was placed on each slide and examined using oil immersion at 400X magnification. The fetal cells were differentiated from the maternal cells based on the intensity and intracellular distribution of pink staining. Each field had an approximate of 200 cells thus 10 fields were examined to give a total cell count of about 2000. The number of fetal cells per slide was then expressed as a percentage of the 2000 cells counted.

To calculate the amount of fetal blood within the maternal circulation, the American Association of Blood Banks formula of 2008 was used:

FMH (ml) = (% of fetal cells by Kleihauer Betke/100) x 5000

The amount of anti-D immunoglobulin required to neutralize the fetal cells was then calculated taking into consideration that 10µg neutralizes 1ml of fetal whole blood (Crowther, 2010).

3.8.1 Biosafety and cross infection control measures

Blood sample handling including collection, storage, transport to the lab, processing and disposal following analysis was done in consultation with a qualified laboratory technologist. This was done to ensure adherence to international biosafety protocols, prevent contamination and ensure sample viability. Personal protective equipment were used as per lab biosafety protocols during the handling of samples and reagents.

3.9 Data reliability and validity

To ensure data reliability and validity, the following measures were taken:

- Research assistants were trained on how to administer the questionnaire and how to optimize sample collection, labelling and storage.
- Questionnaires and sample bottles were counter checked to ensure that the serial numbers tally.
- The fridge used for storage of blood samples prior to transport to the lab was set at 4°C and was checked daily to ensure the set temperature was not altered.
- To determine inter-observer consistency, the results of the Kleihauer Betke test for every tenth participant were re-examined by an independent hematologist with no knowledge of the study.
- Entry of data into the database was reviewed to avoid duplication of data.

3.10 Data management and analysis

The data was entered into an encrypted computer Microsoft Access database which was later exported to R Core Team (2017), a language and environment for statistical computing, for analysis. Access to the data was restricted to the main investigator. Back-ups were made using encrypted memory disks to protect against loss of data.

Categorical variables such as education level, marital status, occupation, and the monthly income category were summarized using frequencies and the corresponding percentages. Continuous variables such as age, duration of pregnancy were summarized using mean and the corresponding standard deviation (SD). Other continuous variables such as fetomaternal hemorrhage, and amount of anti-D dose given were summarized using median and the corresponding interquartile range (IQR) (i.e lower and upper quartiles). Continuous variables were assessed for Gaussian assumptions using histograms and normal probability plots. If the Gaussian assumptions were holding the variable was summarized using mean and SD, if not, median and IQR were used to summarize them.

The prevalence of Rhesus negative mothers delivering at the Moi Teaching and Referral Hospital was computed as the number of Rhesus negative mothers who delivered divided by the total number of deliveries during the study period. The corresponding 95% confidence intervals (95% CI) was reported.

Results were presented using tables and graphs.

3.11 Ethical considerations

1. IREC approval was secured before the study began. (Appendix 5)
2. Permission to conduct the research was granted by the hospital management. (Appendix 4)

3. Informed consent was obtained from each study participant prior to enrollment into the study. (Appendix 1)
4. Privacy and confidentiality was ensured by consenting and interviewing participants in private, storing questionnaires and lab results in a locked cabinet and databases protected with passwords.

CHAPTER FOUR: RESULTS

4.1 Prevalence

A total of 4552 mothers delivered at the Moi Teaching and Referral Hospital during the months of April 2017– September 2017. Of the 4552, 143 were Rhesus negative thus giving a prevalence of 3.1% (95% CI: 2.7, 3.7).

4.2 Socio-demographics

During the study period, 143 Rhesus negative mothers delivered at RMBH. Of these, 99 fit the eligibility criteria and were sampled for inclusion in the study.

The mean (SD) age was 26.4 (SD: 5.4) years with a range of 18-42 years.

Up to 77.8% of the study participants were married and one fifth (21.2%) single. One quarter (24.2%) had a college level of education and 53.5% had a secondary level of education. Detailed socio-demographic characteristics are as shown in table 1.

Table 1: Socio-demographic characteristics of the participants

Variable	N	Mean (SD) or n (%)
Age (Years)	99	26.4 (5.4)
Range (Min. – Max.)		18.0 – 42.0
Marital status		
Single		21 (21.2%)
Married	99	77 (77.8%)
Divorced		1 (1.0%)
Education level		
Primary		22 (22.2%)
Secondary	99	53 (53.5%)
College		24 (24.2%)
Occupation		
Unemployed		55 (55.6%)
Self-employed	99	33 (33.3%)
Employed		11 (11.1%)
Monthly Household income (Kenya Shillings)		
< 10,000	99	69 (69.7%)
≥ 10,000		30 (30.3%)

More than half (55.6%) of the study participants were unemployed.

The monthly household income was less than Kenya Shillings 10,000 for majority (69.7%) of the study participants.

4.3 Obstetrical characteristics

The participants' obstetric information is as shown in Table 2.

Table 2: Obstetric characteristics of study participants

Variable	N	Mean (SD) or n (%)
Gestation at delivery	99	39 (1.9)
Range (Min. – Max.)(weeks)		33 – 43
Gravidity	99	2.1 (1.1)
Range (Min. – Max.)		1.0 – 7.0
One		34 (34.3%)
Two		31 (31.3%)
Three	99	24 (24.2%)
Four or more		10 (10.1%)
Blood Group		
A-		26 (26.3%)
B-	99	22 (22.2%)
AB-		2 (2.0%)
O-		49 (49.5%)
Received anti-D at the 28th week of pregnancy	99	22 (22.2%)
Had elevated blood pressure	99	5 (5.1%)
Severity of the blood pressure	5	
Mild		3 (60.0%)
Severe		2 (40.0%)
Mode of delivery		
Assisted vaginal delivery		1 (1.0%)
Caesarean delivery	99	31 (31.3%)
Spontaneous vaginal delivery		67 (67.7%)
Had the placenta removed manually	99	4 (4.0%)
Pregnancy outcome		
Fresh stillbirth		1 (1.0%)
Single live birth	99	96 (97.0%)
Twins live birth		2 (2.0%)

The average duration of the pregnancy was 39 weeks (SD: 1.9) days with a minimum and maximum of 33.7 and 43 weeks respectively.

The mothers had carried an average of 2.1 (SD: 1.1) pregnancies to viability. The least number of pregnancies carried was 1 and the most was 7.

Data on blood group was captured and the findings show that the most prevalent blood group was O negative at 49.5% and the least prevalent was AB negative at 2.0%.

One fifth (22.2%) of the mothers had received routine antenatal anti-D prophylaxis at 28 weeks gestation.

None of the study participants reported a history of blunt abdominal trauma or abnormal per vagina bleeding during their pregnancy.

With regards to mode of delivery, two thirds had a spontaneous vaginal delivery while 31.3% had caesarean delivery. Only one study participant had an assisted vaginal delivery.

Manual removal of placenta was employed in 4.0% of the mothers.

Of all the deliveries, only one fresh still birth occurred (1.0%) and of the live births, 2.0% were twins.

4.4 Fetomaternal hemorrhage

The results of the Kleihauer Betke test as calculated using AABB formula is as shown below:

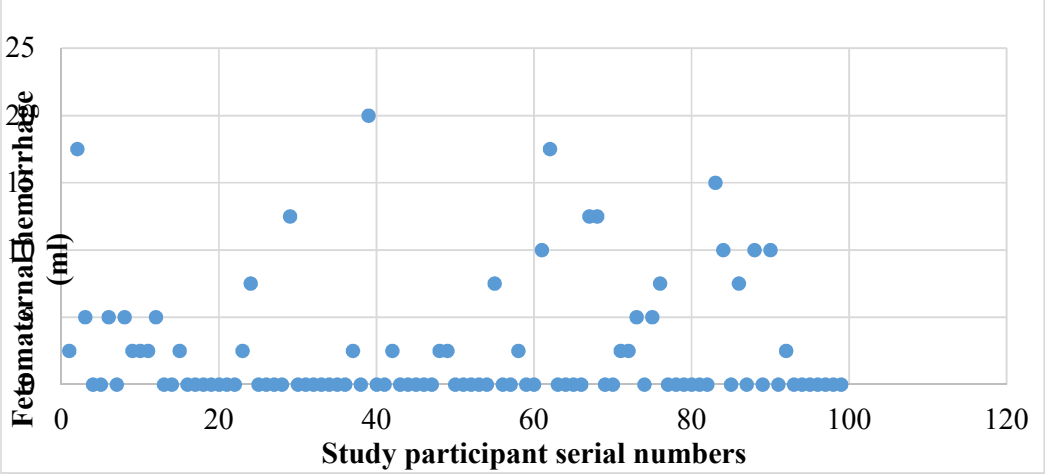

Fig 2: Scatter graph showing individual KHB results

Most of the study participants (64/99) did not have demonstrable FMH. Those that had FMH identified by the KHB test were 35.4% (35/99). The median FMH among the 35 mothers was 5.0 (IQR: 2.5, 10.0) ml with a range of 2.5-20mls.

The distribution of the participants who had fetomaternal hemorrhage by the size of fetomaternal hemorrhage is shown in figure 3.

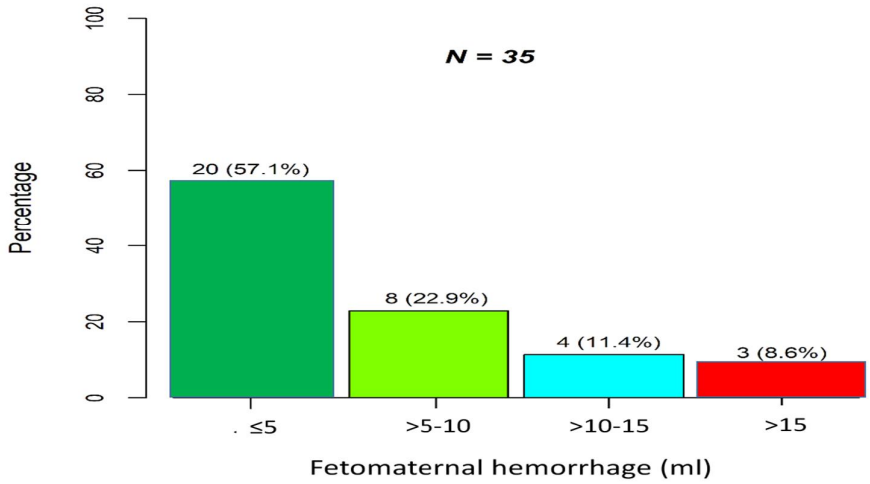

Figure 3: Size of fetomaternal hemorrhage among those who experienced FMH

Fetomaternal hemorrhage of less than 10ml was seen in 68.6% (24/35). The remaining 11 had FMH ranging between 10ml-20ml, the distribution of which is as shown below in table 3.

Table 3: Distribution of FMH ≥ 10ml among study participants

Size of FMH	*No. of participants (n=11)*
10.0ml	4
12.5ml	3
15.0ml	1
17.5ml	2
20.0ml	1

Large FMH, defined as that above 15ml, was only noted in 3 study participants. The largest size of FMH was 20ml of fetal whole blood. This was seen in a 29 year old study

participant who had undergone an emergency caesarian section, at term, for prolonged labour. The outcome in this case was a single live infant with a good APGAR score. Excessive FMH, defined as that exceeding 30ml, was not detected in any of the study participants.

4.5 Anti-D Immunoglobulin dose

The amount of anti-D immunoglobulin that would have been required by the study participants was calculated. The calculation was done taking into consideration that 10µg of anti-D immunoglobulin is required for every 1ml of fetal whole blood.

The calculated amount of anti-D that would have been required against the size of FMH is as shown in figure 4.

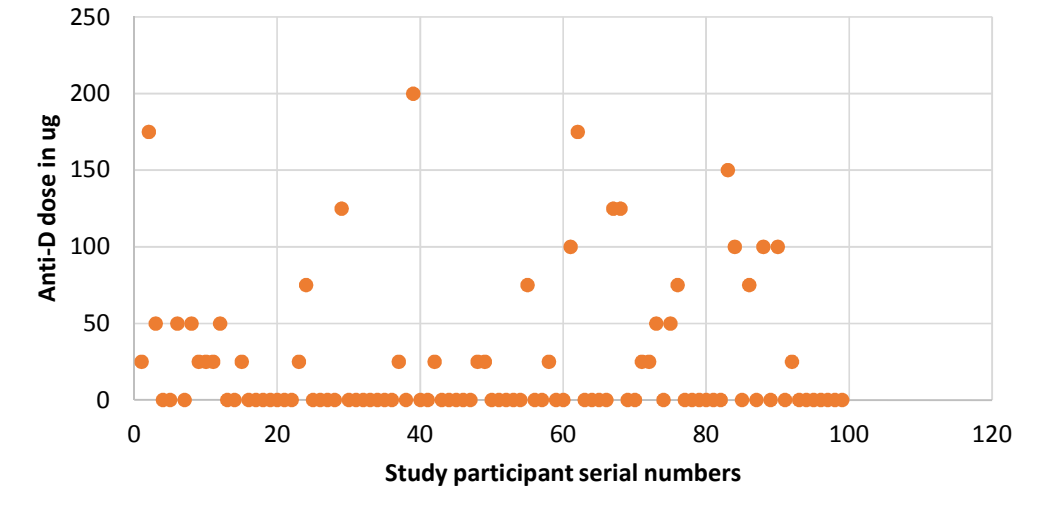

Figure 4: Scatter graph showing individualized Anti-D doses

The median corresponding anti-D immunoglobulin dose that ought to have been administered to those who had FMH was 50.0 (IQR: 25.0, 100.0) µg with a minimum and a maximum of 25.0 and 200.0 ug respectively. This is shown in the table below.

Table 4: Fetomaternal hemorrhage and anti-D dosage

Variable	N	Median (IQR) or n (%)
Size of fetomaternal hemorrhage (ml) Range (Min. – Max.)	35	5.0 (2.5, 10.0) 2.5 – 20.0
Anti-D dose the participant would have required (ug) Range (Min. – Max.)	35	50.0 (25.0, 100.0) 25.0 – 200.0

CHAPTER FIVE: DISCUSSION

5.1 Prevalence of Rhesus negative mothers

The prevalence of Rhesus negative blood group among mothers delivering in our maternity unit was found to be 3.1%. This finding is consistent with a study by Mwangi J, in which he found that 3% of pregnant patients attended to at the Aga Khan University hospital in Nairobi were Rhesus negative (Mwangi J, 1999). Similar findings have also been demonstrated in Uganda in which the prevalence was found to be 3.8% (Mbalibulha *et al,* 2018) and in Nigeria where the prevalence was 4.5% (Okeke *et al,* 2012). The similarities in the prevalence could be because these studies were done in similar geographical regions in which majority of the population are of the same race. Different findings were reported by Szczepura in the UK (Szczepura *et al, 2011)* and Haas in Netherlands (de Haas *et al,* 2016) in which the prevalence was 10% and 15% respectively. Rhesus negativity tends to have a racial predilection in its distribution such that it is higher in Caucasian populations and lower in other ethnic groups (Freeman, 2009). This could explain the higher prevalence noted in the two studies by Szczepura and Haas.

5.2 Fetomaternal hemorrhage

Entry of fetal blood into the maternal circulation occurs in approximately 75% of pregnancies but is only a concern if the size is large enough to cause fetal hemodynamic compromise or in instances of Rhesus incompatibility between the mother and fetus. FMH sufficient to cause isoimmunisation occurs most commonly at birth in 15-50% of pregnancies. (ACOG, Clinical guidelines Number 75, 2006).

Fetomaternal hemorrhage in this study was detected in 35.4% of the study participants. This finding is in line with Mulandi's study that detected FMH in 35.9%

of Rhesus negative mothers delivering in KNH (Mulandi, 1983). This could be due to the similarity in the demographic regions in which the studies were conducted.

However, a study in India demonstrated FMH in 92% of patients (Savithrisowmya *et al,* 2008) while in Ethiopia FMH was reported in 52% (Urgessa *et al,*2014) . The large variation seen between this study and that of Savithrisowmya could be the differences in sample size as he had a much smaller sample size of 25. In addition, the variations could be due to different clinical factors that have a bearing on the occurrence of FMH such as: mode of delivery, the use of fundal pressure as reported in the Indian study, surgical technique and mode of placental delivery.

In this study, 89.9% of the 99 Rhesus negative patients in whom FMH was tested, had a FMH of less than 10ml which is similar to the findings of Urgessa of 92.5% (Urgessa *et al,* 2014) and David of 94.1% (David *et al,* 2004). Excessive FMH (exceeding 30ml) was not detected in this study. This is in congruence with a study by Kumari in 2017. Supporting this low occurrence is a study in Ethiopia that showed excessive FMH in only 1.3% (Urgessa *et al,* 2014). The occurrence of excessive FMH is considered rare, complicating about 0.7% of normal deliveries (Greer, 2003). The similarities in the size of FMH have been observed despite differences in sample size and study types. This shows that majority of patients across different populations that have FMH have a minimal size of FMH.

The age difference between those that experienced FMH and those that did not was noted to be statistically significant, mean age: 29.0 (SD: 6.4) years vs. 25.1 (SD: 4.2) years, p-value = 0.002. While many studies on factors affecting FMH exist, their individual conclusions are contradictory. In line with this study is one by Kumari in

2017 that established that as the maternal age increases, the risk of demonstrable FMH also increases (Kumari, 2017). However, most studies such as those by Studnickova *et al* in 2012 and Stroustrop in 2016 showed that maternal age does not affect the incidence of fetomaternal hemorrhage.

All patients that had manual removal of placenta (4/99) had FMH. However the numbers were too few to allow for further analysis. Kumari *et al* reported that patients who had manual removal of placenta were more likely to have FMH (Kumari, 2017). Manual removal of placenta is a known risk factor for occurrence of fetomaternal hemorrhage with a plausible explanation being that tearing of the placental villi during extraction favors entry of fetal cells into the maternal circulation.

When comparing mode of delivery, there were no significant differences in the mode of delivery between those who had FMH and those who did not. A study by Adeniji *et al* in 2008 in Nigeria similarly found that the mode of delivery did not have a significant effect on the occurrence of FMH.

5.3 Anti-D dose requirements

Anti-D dosing is ideally based on fetomaternal hemorrhage, taking into consideration that 10µg would neutralize 1ml of fetal whole blood or 0.5ml of packed fetal red cells. (Crowther, 2000).

A systematic review by Cochrane established that evidence on the optimal amount of anti-D for postpartum prophylaxis is limited (Crowther *et al,* 2013). The recommendations on the ideal dose vary in different countries and is usually guided by their respective professional associations. In Kenya, the guidelines recommend

administration of a standard dose of 300µg (National guidelines for quality obstetrics and perinatal care, 2011), which is similar to guidelines in the United States of America (ACOG Practice Bulletin No. 4, 2010).

FMH was demonstrated in only 35.4% (35) of the 99 sampled Rhesus negative mothers. Of the 35 mothers, only 11 had FMH of 10ml and above. Taking this into consideration, individual doses of 100µg of anti-D would have been sufficient for prophylaxis in majority (24) of the 35 mothers who had FMH.

If we include those who did not have any detectable FMH, it means that individual doses of 100µg of anti-D would have been sufficient for prophylaxis in 89.9% of all the study participants.

These findings are in agreement with an Ethiopian study that showed that 100µg would have been sufficient to neutralize FMH in majority (92.5%) of Rhesus negative mothers after delivery (Urgessa *et al,* 2014). Similar observations were reported in a prospective study in a teaching hospital in Germany which demonstrated that 100µg of anti-D would have been sufficient postpartum prophylaxis for 94.2% (David *et al,* 2004).

Of the minority of patients that had FMH of 10ml or more, the range was 10ml-20ml. Four had FMH of 10ml, three had 12.5ml, one had 15ml, two had 17.5ml and one had 20ml.While these patients would have required more than 100µg of anti-D, the maximum dose would have been 200µg which is still lower than the standard 300µg recommended.

A retrospective multicenter study by Augustson *et al* in 2006 established that only 0.4% of the 5,148 study participants would have required more than 100µg of anti-D for postpartum prophylaxis. He further established that individualized anti-D dose based on FMH would have resulted in a 30% reduction in costs on Anti-D immunoglobulin purchase.

These findings imply that that the blanket anti-D dose of 300 micrograms routinely administered was largely in excess of what would be needed to cover the measured FMH.

CHAPTER SIX
CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

1. The prevalence of Rhesus negative mothers delivering in MTRH is 3.1% which is similar to the regional prevalence of Rhesus negativity.
2. Fetomaternal hemorrhage in this study was undetectable or small (less than 15ml) in most study participants.
3. Majority of the cases of FMH could have been neutralized by 100μg of Anti-D.

6.2 Recommendations

Quantification of FMH and individualized dosing of anti-D immunoglobulin in unsensitised Rhesus negative mothers after delivery is recommended.

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APPENDICES

Appendix 1: Consent Form

My name is Dr. Fatma A. Agil. I am currently pursuing a Masters Degree at Moi University. A requirement of this course is to do a dissertation, I chose to study on quantification of fetomaternal haemorrhage and its application in anti-d dosing in rhesus negative mothers delivering in Moi Teaching and Referral Hospital. This research is about calculating the amount of your blood that mixes with your baby's blood and this will help us know if the Anti-D we are giving you is adequate or not. In order to do this I will require to take a small sample of blood and ask you questions about your socio demographic and reproductive health characteristics. You are free to respond or choose not to respond to some of the questions that you may find inconvenient to you. You may also decline to give a sample of blood.

RISK-BENEFIT ASSSESMENT

Your participation in this study will not affect in any way the treatment plan that your doctors have planned for you. Your decision to participate will not change or prejudice your care in the hospital. This study is a minimal risk study and has more benefits than harm. The benefit of this study is that it will determine the right dosage of anti-D required for you as an individual to prevent your body from mounting an immune response to your baby in future pregnancies. The information obtained may also be used to change clinical practice within our setup to improve management of rhesus negative women during pregnancy.

The potential risk you face as a study participant is that you may experience minor pain as a sample of blood is being drawn. There is no legal risk in this study since it is a voluntary participation with adequate informed consent. Information gathered will be treated with utmost confidentiality; your identity will be protected (your name will not

be used and you will be identified with a number, only known to me and my immediate assistant).

The information obtained will be used to improve services in the clinic and may be published in medical journals and/or presented in scientific symposia (both local and international).

This proposal has been reviewed and approved by IREC, which is a committee whose task it is to make sure that research participants are protected from harm.

For any question or clarification, please do not hesitate to contact me on 0723677727 or contact the chairperson of IREC, MOI TEACHING AND REFERRAL HOSPITAL BUILDING, second floor room 219. Phone number 0787723677.

Certificate of Consent

I have read the above information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate as a participant in this research.

Name of Participant_____

Signature of Participant _____

Date _____

If Guardian

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Name of witness_____ AND Thumb print of participant

Signature of witness _____

Date _____

Statement by the researcher/person taking consent

I have accurately read out the information to the potential participant, and to the best of my ability made sure that the participant understands what will be done.

I confirm that the participant was given an opportunity to ask questions about the study, and all the questions asked by the participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

Name of Researcher/person taking the consent_____

Signature of Researcher /person taking the consent_____

Date _____

KIAMBATISHI 1B: CHETI CHA KUTOA IDHINI KWA HIARI

Jina langu ni Dkt .Fatma A. Agil na ninasomea Shahada ya Uzamili katika Chuo Kikuu cha Moi. Moja ya mahitaji ya masomo haya nikufanya tasnifu, nami nimeamua kuchunguza kuhusu kikundi cha damu kinachojulikana kama “Rhesus Negative”. Nitakuuliza maswali juu ya kijamii na afya yako ya uzazi. Pia nitahitaji kuchukua kiwango kidogo cha damu kupima kiasi damu yako na ya mtoto imechanganyika ili kujua kama kiwango cha dawa tunayokupatia inafaa. Una uhuru kujibu au kutojibu baadhi ya maswali ambayo hayakupendezi au kukataa kutolewa damu.

UCHUNGUZI WA HATARI NA MANUFAA

Ushirikiano wako katika utafiti huu hautoathiri kwa namna yoyote mpangilio wa tiba yako uliyopangiwa na madaktari wako. Uamuzi wako wa kushiriki katika utafiti huu hautobadilisha wala hautoathiri huduma yako katika hospitali hii. Utafiti huu una faida zaidi kuliko madhara. Faida ya uchunguzi huu utatusaidia kupata uamuzi wa kipimo sahihi cha dawa inayohitajika kupambana na “D antigen” ili kuzuia kinga ya mwili wako mwenyewe usiharibu mtoto wako katika mimba za baadaye. Matokeo yatakayo patikana katika uchunguzi huu unaweza kubadilisha desturi na kuboresha huduma kwa wanawake wajawazito wenye damu iliyo “Rhesus negative.”

Unaposhiriki katika uatafiti huu huwenda ukapata uchungu kidogo wakati wa kutolewa damu kama sampuli kwa uchunguzi huu. Hakuna maafa au hatari ya kisheria katika utafiti huu kwa kuwa ni ushirikiano wa hiari na ridhaa na maelezo ya kueleweka.

Taarifa zitakazo patikana na kukusanywa zitabaki kuwa ni siri itakayo hifadhiwa; na jina lako halitotumiwa utatambuliwa kwa nambari utakayo tumiwa na mimi na msaidiziwangu wa karibu mno.

Taarifa zitakazopatikana zitatumika kuboresha huduma katika zahanati na inaweza kuchapishwa katika jarida la tiba na / au iliyotolewa katika makongamano ya kisayansi (ndani na kimataifa)Pendekezo la utafiti huu umeidhinishwa na IREC, ambayo ni kamati lenye jukumu la kulinda haki na usalama wa washiriki wa utafiti.

Kwa ufafanuzi au swali lolote, tafadhali usisite kuwasiliana nami kwenye nambari 0723677727 au na Mwenyekiti wa IREC,JENGO LA MOI TEACHING AND REFERAL HOSPITAL,OROFA YA PILI -CHUMBA 219. NAMBARI YA SIMU-0787723677.

Cheti cha uthibitisho wa hiari

Nimesoma ujumbe uliotangulia, au yameelezwa kwangu. Nimepata nafasi ya kuuliza maswali ambayo yamejibiwa hadi nikaridhika. Kwa hiari, ninajijumuisha kwa utafiti huu kama mshiriki.

Jina la Mshiriki_____

Sahihi ya Mshiriki _____

Tarehe _____

Ikiwa ni msaidizi

Nimeshuhudia kusomewa sahihi kwa cheti hiki kwa mshiriki mtarajiwa, naye amepewa nafasi wa kuuliza maswali. Ninathibithisha kwamba mshiriki ametoa hiari yako kwa kuchagua mwenyewe.

Jina la shahidi_____ PAMOJA NA Chapa ya kidole gumbe cha mshiriki

Sahihi ya shahidi _____

Tarehe _____

Taarifa ya mtafiti/ anayechukua hiari

Nimeusoma ujumbe kwenye karatasi lote sahihi kwa mshiriki mtarajiwa kwa uwezo wangu wote na kuhakikisha kuwa ameelewa yote yatakayofanyika.

Ninathibitisha kuwa mshiriki amepewa nafasi ya kuuliza maswali yote na yote kujibiwa vyema jinsi ya uwezo wangu. Nathibitisha kuwa hajashurutishwa kutoa hiari yake, na hiari yenyewe imetolewa kwa uhuru na kupenda mwenyewe.

Jina la mtafiti/ anayechukua hiari_____

Tarehe _____

Appendix 2: Questionnaire (English)
SERIAL NO…….. **DATE ……..**

SECTION A: Mother's Socio-Demographic Characteristics

1. Age: ……. 2. Parity:…….

3. Marital status: [ ] married [ ] single [ ] divorced [ ] widowed [ ] cohabitation

4. Your highest level of education: [ ] no education [ ] primary school [ ] secondary school [ ] college [ ] other, specify……..

5. What is your main occupation? .....

6. What is the monthly household income? [ ] KShs.10, 000 or less [ ] more than KShs.10,000

SECTION B: Mother's Reproductive-Obstetrical information (on the current delivery)

7. 1st day of last menses: …… 8.Gestation by date:……

9. Blood group……

10. Did you receive anti-D during this pregnancy? [ ] Yes [ ] No

11.If yes, how many times?..... At what gestation?.....

12. Did you experience any abnormal per vaginal bleeding during this pregnancy?

[ ] Yes [ ] No

13. During the course of your pregnancy, did you experience any form of blunt abdominal trauma?

[ ] Yes [ ] No

14. Did you have elevated blood pressures requiring treatment during this pregnancy?

[ ] Yes [ ] No

15. If yes , what was it classified as? [ ] Mild [ ] Severe

16. What was the mode of delivery for this pregnancy? [ ] SVD [ ] CS [ ] AVD

17. Was manual removal of placenta done? [ ] Yes [ ] No

18. What was the number of infants delivered in this pregnancy?

[ ] Singleton [ ]Twins [ ] More than two

19. What was the outcome of this pregnancy? [ ] Live birth [ ] Still birth

20. Were there any features of hydrops fetalis? [ ]Yes [ ] No

SECTION C: MOTHER'S PAST MEDICAL HISTORY

21. Have you ever received a blood transfusion? [ ] Yes [ ] No

22. Have you ever been treated for any hematologic disease? [ ] Yes [ ] No

23. If yes, please explain further……

Thank you for your cooperation.

SECTION D:

24. Number of fetal cells per high power field …….

25. Number of maternal cells per high power field ……..

26. Amount of fetomaternal hemorrhage: …….

27. Amount of Anti-D required: …….

Appendix 2b: Dodoso (Kiswahili)

NAMBARI…….. **TAREHE..…….**

SEHEMU A: Tabia ya kijamii na idadi ya watu ya Mama

1. Umri: ……. 2. Kizazi……

3. Hali ya ndoa [ ] Nimeolewa [ ] sijaolewa [ ] Mtalaka [ ] Mjane [ ] Naishi na mchumba

4. Kiwango cha juu cha elimu: [ ] sijasoma [ ] shule ya msingi [ ] shule ya upili

[ ] chuo kikuu [ ] nyingine (taja)……..

5. Unafanya kazi aina gani? .....

6.Ni kiasi kipi cha mapato mnaopata nyumbani kwa mwezi? [ ] Chini elfu kumi [ ] zaidi ya elfu kumi

SEHEMU B: habari ya mama kuhusu uzazi na ujaa uzito (kulingana na kujifungua hivi majuzi)

7. Tarehe ya siku ya kwanza ya hedhi ya mwisho……

8. Muda wa ujauzito……

9. Kikundi cha damu…….

10. Umewahi kupata chanjo cha Anti-D kwa mimba hii? [ ] Ndio [ ] La

11. Ikiwa umepata, umepata mara ngapi? ……Wakati gani wa ujauzito? ……..

12. Kuna wakati wowote katika mimba hii ulitokwa na damu njia ya uzazi? [ ] Ndio [ ] La

13. Kuna wakati wowote katika mimba hii ulipata jeraha la tumbo? [ ] Ndio [ ] La

14. Je, ulikuwa na shida ya presha iliyolazimisha dawa katika hii mimba? [ ] Ndio [ ] La

15. Ikiwa ulikuwa na shida ya presha, ilikuwa ya kiwango gani? [ ] Chini [ ] Juu

16. Mbinu ya kujifungua: [ ] kupasuliwa [ ] kupitia kwa uke [ ] mtoto kuvutwa na chombo

17. Je, kizalio kilikwama na kuhitaji usaidizi wowote kutoka? [ ] Ndio [ ] La

18. Ulizaa watoto wangapi wakati huu? [ ] Mmoja [ ] Mapacha [ ] Zaidi ya wawili

19. Mtoto aliyezaliwa alikuwa? [ ] Hai [ ] Mfu

20. Kulikuwa na ishara yoyote ya mtoto kufura mwili? [ ] Ndio [ ] La

SEHEMU C : Historia ya matibabu ya zamani

21. Umewahi kuongezwa damu mbeleni? [ ] Ndio [ ]La

22. Umewahi kutibiwa ugonjwa wowote wa damu? [ ]Ndio [ ] La

23. Kama umewai, tafadhali fafanua……

Ahsante kwa wakati wako

SEHEMU D:

24. Kiasi cha damu kilichochanganyika: ……..

25. Kiwango cha Anti-D kinachofaa: ……

Appendix 3: Sigma Aldrich Fetal Hemoglobin Kit Working Instructions for Lancet Hematology Laboratory

SIGMA-ALDRICH®

FETAL HEMOGLOBIN
(Procedure No. 285)

INTENDED USE

Fetal Hemoglobin reagents are for the acid elution, semi-quantitative determination of fetal hemoglobin in blood smears. Fetal Hemoglobin stain reagents are for "In Vitro Diagnostic Use."

As early as 1864, Korber[1] recognized that the hemoglobin of the fetus was more resistant to alkali denaturation than that of the adult. Advances in techniques for protein isolation and characterization led to the discovery that there are several distinguishing properties that make it possible to differentiate fetal from adult hemoglobin. Among these is the resistance of fetal hemoglobin (hemoglobin F) to acid elution. When blood smears are immersed in acid buffer, for example, adult hemoglobin is eluted from the erythrocytes, whereas fetal hemoglobin is not. If blood smears are treated in this manner and subsequently stained, erythrocytes having hemoglobin F will take up the stain, while those containing only adult hemoglobin appear as "ghosts".

The slide technique for demonstrating fetal hemoglobin in terms of its resistance to acid elution was originally proposed by Kleihauer et al.,[2] and later modified by Shepard et al.[3] The Sigma procedure represents a further improvement in this approach as described by Oski and Naiman.[4]

Fetal hemoglobin estimations are sometimes made to determine possible hemorrhage in the newborn, particularly in cases where there are signs of rectal bleeding. Hemoglobin F assay is also applied to adults as an aid in diagnosing certain types of anemia. For example, from 10–90% fetal hemoglobin is encountered in patients with thalassemia major. Moreover, small increases of fetal blood pigment are usually observed in patients with sickle cell disease.

It is becoming increasingly common in cases of Rh incompatibility to suppress immune reactions to red blood cells entering maternal circulation from the fetus. The amount of specific gamma globulin, containing anti Rh(D) to be administered, is calculated by assessing the magnitude of fetal-maternal hemorrhage.[5]

According to described technique, blood smears, which have been properly dried and fixed, are immersed in a citrate buffer pH 3.3 at 37°C. Adult hemoglobin A (HbA) dissolves out of the cells, whereas fetal hemoglobin (HbF) which is acid resistant, remains intracellular and can be stained for microscopic examination.

REAGENTS

CITRATE PHOSPHATE BUFFER CONCENTRATE, Catalog No. 2851
Sodium citrate, 0.7 mol/L, and sodium phosphate, 0.6 mol/L.
ACID HEMATOXYLIN SOLUTION, Catalog No. 2852
Hematoxylin, certified, 1 g/L, aluminum ammonium sulfate, sodium iodate and stabilizers, pH 3.3.
EOSIN B SOLUTION, Catalog No. 2853
Eosin B, 0.1%, aqueous solution. Sodium azide, 0.1%, added as preservative.
STORAGE AND STABILITY:

Store Citrate Phosphate Buffer Concentrate in refrigerator (2–8°C). Discard if there is evidence of microbial growth.

Store Citrate Phosphate Buffer Solution in refrigerator (2–8°C). Stable for 2 weeks. Use a fresh aliquot each day. Discard if there is evidence of microbial growth.

Store Acid Hematoxylin Solution and Eosin B Solution at room temperature (18–26°C). Solutions may be reused if they are stored in tightly sealed staining jars in subdued light.

Ethanol fixative should be stored at room temperature. Store tightly sealed and as a flammable liquid. Solution may be reused, but should be discarded if fixation is not adequate.

DETERIORATION:

Discard Acid Hematoxylin Solution when the time required for suitable staining exceeds 8 minutes.

PREPARATION:

Citrate Phosphate Buffer Solution is prepared by diluting 1 volume of Citrate Phosphate Buffer Concentrate with 9 volumes of water.

Acid Hematoxylin Solution, Eosin B Solution and Ethanol Fixative are ready to use.

PRECAUTIONS:

Normal precautions exercised in handling laboratory reagents should be followed. Dispose of waste observing all local, state, provincial or national regulations. Refer to Material Safety Data Sheet and product labeling for any updated risk, hazard or safety information.

PROCEDURE

SPECIMEN COLLECTION:

It is recommended that specimen collection be carried out in accordance with CLSI document M29-A3. No known test method can offer complete assurance that blood samples or tissue will not transmit infection. Therefore, all blood derivatives or tissue specimens should be considered potentially infectious.

Either capillary or venous blood may be used. Capillary blood may be transferred directly to a clean microscope slide. Venous blood should be added to a tube containing EDTA or oxalate. For convenience, use 1–2 drops of 2% EDTA Solution, Catalog No. 2854, per mL of blood (1 drop = 1 mg). Although blood-EDTA mixtures have been reported to be satisfactory for use up to 2 weeks when refrigerated,[3] other studies have concluded that such mixtures should be tested promptly.[6] Smears should be prepared within 24 hours from blood collected in oxalate.[7] Using samples from the newborn, it is recommended that the blood be diluted with 0.85% saline, since such specimens have a high content of HbF. Blood smears are not stable and must be tested immediately after preparation.

SPECIAL MATERIALS REQUIRED BUT NOT PROVIDED:
Microscope
Microscope slides, cover slips
Staining rack/Coplin jars
Water bath, 37°C
Ethanol Fixative, Catalog No. 2858, 80% v/v ethyl alcohol

NOTES:

For quality control purposes, it is recommended that blood from a normal adult (HbA) and from a newborn or infant (HbF) be included in each series of tests. Barr and Shafer[8] report that fixed positive control slides from cord blood in EDTA can be preserved up to 1 year at –20°C, in a sealed cardboard box. However, EDTA negative controls do not elute completely after being stored for more than 2 months at –20°C. These investigators suggest preparing positive and negative smears on the same slide; thus, providing clear and rapid contrasts as reference in reading test slides.

Normal Ranges[9]

Age	Fetal Hemoglobin (%)
At Birth	50–90
< 2 years	0–4
> 2 years	0–2

Excessive values are observed in:
- Aplastic anemia[3,9]
- Erythremic myelosis[9]
- Hemoglobin H disease[9]
- Hereditary persistence of hemoglobin F[3,10]
- Hereditary spherocytic anemia[9]
- Thalassemia major (40–90% fetal hemoglobin)[3]
- Thalassemia minor (5–10% fetal hemoglobin)[3,9]
- Sickle cell anemia[3,9]

The data obtained from this procedure serves only as an aid to diagnosis and should be reviewed in conjuction with other clinical diagnostic tests or information.

PROCEDURE:

1. Citrate Phosphate Buffer Solution should be warmed to 37°C in a Coplin jar or staining dish.
2. Using clean, labeled microscope slides, make thin blood smears. Prepare CONTROL slides using positive HbF blood (cord-blood) and normal adult blood. Air dry approximately 10 minutes.
3. Fix slides by immersing in Ethanol Fixative for 5 minutes, rinse thoroughly with tap water and air dry.
4. Immerse TEST and CONTROL slides in pre-warmed Citrate Phosphate Buffer Solution at 37°C for 5 minutes. Agitate after 1 and 3 minutes of immersion. Degree of agitation may be varied to achieve most desirable results. Rinse thoroughly with distilled water and air dry **completely** to avoid staining artifacts.
5. Stain the slides for 3 minutes in Acid Hematoxylin Solution. Rinse slides with distilled water and shake off excess water.
6. Counterstain slides for 4 minutes in 0.1% Eosin B Solution. Rinse thoroughly with distilled water and air dry.
7. Place **dry** coverslip on slide and examine using oil immersion (1000X). The absence of HbF is evident by the presence of ghost cells while retained HbF causes cells to appear bright red. Do **not** apply oil directly to slide.
   NOTE: The 400X magnification may be used, but the resulting larger field may be more difficult to count.

PERFORMANCE CHARACTERISTICS

The proportion of erythrocytes containing fetal hemoglobin may be estimated several ways. When studying maternal blood for evidence of HbF-containing cells, Oski and Naiman[4] recommended the following:

1. Count total number of erythrocytes in 5 fields and determine the average number per field.
2. Count the number of deeply stained HbF-containing erythrocytes in about 30 fields and determine the average number per field.
3. Calculate percentage of HbF-containing erythrocytes on the basis of the total number of erythrocytes per field.

Results are reported as the percent HbF present.

Sensitivity studies: According to Oski and Naiman[4] this method is capable of detecting as little as 0.1 mL of fetal blood in maternal circulation.

Reproducibility studies: Using a series of fresh blood specimens, replicate slides were prepared from each and treated with several different lots of stain on separate occasions.[6] Microscopic examination revealed essentially identical results with each blood sample.

Correlation studies: Mixtures of cord blood and compatible adult blood were prepared to yield specimens with HbF concentrations ranging from 26–66%.[6] The blood mixtures were examined by the described technique and assayed chemically by an alkali denaturation method.[10] The percent HbF values showed an average difference of about 7% between methods.

If observed results vary from expected results, please contact Sigma-Aldrich Technical Service for assistance.

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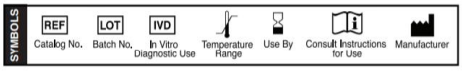
Procedure No. 285
Previous Revision: 2005-01
Revised: 2014-09



EC REP MDSS GmbH
Schiffgraben 41
30175 Hannover, Germany

SIGMA-ALDRICH, INC.
3050 Spruce Street, St. Louis, MO 63103 USA
314-771-5765
Technical Service: 800-325-0250
or e-mail at clintech@sial.com
To Order: 800-325-3010
www.sigma-aldrich.com

Appendix 4: Hospital Approval to Conduct Research

MOI TEACHING AND REFERRAL HOSPITAL

Telephone: 2033471/2/3/4
Fax: 61749
Email: director@mtrh.or.ke
P. O. Box 3
ELDORET

Ref: ELD/MTRH/R.6/VOL.II/2008

28th September, 2016

Dr. Fatma A. Agil,
Moi University,
School of Medicine,
P.O. Box 4606-30100,
ELDORET-KENYA.

RE: APPROVAL TO CONDUCT RESEARCH AT MTRH

Upon obtaining approval from the Institutional Research and Ethics Committee (IREC) to conduct your research proposal titled:-

"Quantification of Feto-Maternal Haemorrhage and Its Application in Anti-D Immunoglobulin Dosing in Rhesus Negative Mothers Delivering at MTRH, Eldoret".

You are hereby permitted to commence your investigation at Moi Teaching and Referral Hospital.

28/09/2016

DR. WILSON ARUASA
CHIEF EXECUTIVE OFFICER
MOI TEACHING AND REFERRAL HOSPITAL

CC - Deputy Director (CS)
- Chief Nurse
- HOD, HRISM

Appendix 5: IREC Formal Approval

INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE (IREC)

MOI TEACHING AND REFERRAL HOSPITAL
P.O. BOX 3
ELDORET
Tel: 33471//2/3

MOI UNIVERSITY
SCHOOL OF MEDICINE
P.O. BOX 4606
ELDORET

Reference: IREC/2016/81
Approval Number: 0001687

28th July, 2016

Dr. Fatma A. Agil,
Moi University,
School of Medicine,
P.O. Box 4606-30100,
ELDORET-KENYA.

INSTITUTIONAL RESEARCH & ETHICS COMMITTEE
28 JUL 2016
APPROVED
P. O. Box 4606-30100 ELDORET

Dear Dr. Agil,

RE: FORMAL APPROVAL

The Institutional Research and Ethics Committee has reviewed your research proposal titled:-

"Quantification of Feto-Maternal Haemorrhage and Its Application in Anti-D Immunoglobulin Dosing in Rhesus Negative Mothers Delivering at MTRH, Eldoret".

Your proposal has been granted a Formal Approval Number: ***FAN: IREC 1687*** on 28th July, 2016. You are therefore permitted to begin your investigations.

Note that this approval is for 1 year; it will thus expire on 27th July, 2017. If it is necessary to continue with this research beyond the expiry date, a request for continuation should be made in writing to IREC Secretariat two months prior to the expiry date.

You are required to submit progress report(s) regularly as dictated by your proposal. Furthermore, you must notify the Committee of any proposal change (s) or amendment (s), serious or unexpected outcomes related to the conduct of the study, or study termination for any reason. The Committee expects to receive a final report at the end of the study.

Sincerely,

PROF. E. WERE
CHAIRMAN
INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE

cc CEO - MTRH Dean - SOP Dean - SOM
Principal - CHS Dean - SON Dean - SOD

Appendix 6: IREC Continuing Approval

INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE (IREC)

MOI TEACHING AND REFERRAL HOSPITAL
P.O. BOX 3
ELDORET
Tel: 33471//2/3

MOI UNIVERSITY
SCHOOL OF MEDICINE
P.O. BOX 4606
ELDORET
Tel: 33471/2/3

Reference: IREC/2016/81
Approval Number: 0001687

28th July, 2017

Dr. Fatma A. Agil,
Moi University,
School of Medicine,
P.O. Box 4606-30100,
ELDORET-KENYA.

INSTITUTIONAL RESEARCH & ETHICS COMMITTEE
28 JUL 2017
APPROVED
P. O. Box 4606-30100 ELDORET

Dear Dr. Agil,

RE: CONTINUING APPROVAL

The Institutional Research and Ethics Committee has reviewed your request for continuing approval for your study titled:-

"Quantification of Feto-Maternal Haemorrhage and Its Application and Anti-D Immunoglobulin Dosing in Rhesus Negative Mothers Delivering at MTRH, Eldoret".

Your request has been granted Approval with effect from 28th July, 2017. You are therefore permitted to continue with your study.

Note that this approval is for 1 year; it will thus expire on 27th July, 2018. If it is necessary to continue with this research beyond the expiry date, a request for continuation should be made in writing to IREC Secretariat two months prior to the expiry date.

You are required to submit progress report(s) regularly as dictated by your proposal. Furthermore, you must notify the Committee of any proposal change (s) or amendment (s), serious or unexpected outcomes related to the conduct of the study, or study termination for any reason. The Committee expects to receive a final report at the end of the study.

Sincerely,

PROF. E. WERE
CHAIRMAN
INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE

cc:
CEO	-	MTRH
Principal	-	CHS
Dean	-	SOM
Dean	-	SPH
Dean	-	SOD
Dean	-	SON