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UNIVERSITY OF GHANA
COLLEGE OF HEALTH SCIENCES

RHESUS D VARIANTS AMONG PREGNANT WOMEN IN THE GREATER ACCRA REGION OF GHANA

BY

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DECLARATION

I, Elvis Baseriyi Batuu, hereby declare that except for reference to other people's work, which I have duly cited, this thesis is the result of an original research work and that the material has not been presented either in whole or in part elsewhere for another degree and all experimental works were performed by me under the supervision of Dr Charles Brown and Dr Michael Mark Addae.

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ABSTRACT

Background: The rhesus (Rh) blood group system is one of the most polymorphic and immunogenic blood group systems known in humans. The common potent Rh antigens reside on the RH polypeptides. The antigenic expression is controlled by the RHD and RHCE genes, linked in tandem on chromosome 1. The D antigen is clinically the most important because of its high immunogenicity and polymorphism. HDFN is perpetuated by foeto-maternal rhesus antigens incompatibility.

Aim: The aim of this study was to identify and classify rhesus D (RH1) variants among pregnant women, that were at a risk of haemolytic disease of the foetus and newborn (HDFN).

Methods: This was a cross sectional study involving 110 pregnant women. The RHD status of participants was confirmed using monoclonal antibodies and weak D expression was confirmed by indirect antiglobulin test. Antibody screening was performed using coombs gel cards. RH primers were used to amplify exon 7 which is identical to both RHCcEe and RHD cDNA, and exon 10 that is specific to the D sequence. PCR multiplex assay using six RHD specific primer sets designed to amplify RHD exons 3, 4, 5, 6, 7 and 9 was used for RHD genotyping.

Results: All the 110 samples were confirmed as RHD negative by serology. Eight (7.3%) out of the 110 RHD negative samples showed the presence of red cell antibodies in the serum. Thirty four (30%) samples had both exon 7 and 10, depicting an RHD positive phenotype, 67 (60%) samples had only exon 7 amplified, denoting RHD negative phenotype and 9 (8.2%) samples, had neither exon 7 nor 10 denoting indeterminate status. None of the six RHD specific exons were amplified from the 34 D+ and 67 D- individuals.

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Conclusion: The rate of alloimmunisation was 7.3% that require RHD immunoglobulin prophylaxis. Thirty percent (30%) of the serological RHD negative samples were molecularly RHD positive hence need to be classified as such. This study shows that not all RHD negative pregnant women require anti-D immunoglobulin prophylaxis.

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DEDICATION

I dedicate this work to the almighty God, my Family, parents, and siblings especially to my late brother Gregory for your encouragement throughout this program.

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LIST OF ABBREVIATIONS

FMH	Foeto-maternal haemorrhage
HbA	Haemoglobin A
HbF	Haemoglobin F
HDFN	Haemolytic disease of foetus and newborn
PCR	Polymerase chain reaction
RBC	Red blood cell
RHD-	Rhesus factor D
SNP	Single nucleotide polymorphism

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CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

The Rhesus blood group system is one of the most polymorphic and immunogenic systems of blood group antigens known in humans. This blood group system is comprise of numerous antigens, principally among them is ‘’RHD’’, ‘’RhC’’, “Rhc”, “RhE” and “Rhe” antigens (Flegel, 2007; Sell *et al.*, 2013).

The Rh factor is a term used to describe the presence of the D antigen, the first antigen that was identified belonging to the Rh blood group system (Huang, 2013), on the surface of erythrocytes. The common potent Rh antigens reside on the RH polypeptides, whose expression is controlled by the RHD and RHCE genes, linked in tandem on chromosome one. The RHD produces the D antigen and RHCE produces the alleles Ce, ce, cE and CE compound antigens. There are about 300 allelic forms of the RHD and RHCE genes named at the molecular level. This polymorphism is due to various pattern of genomic diversification at the RH locus. The predominant mechanism of diversification at the RH locus is coding nucleotide changes and genomic rearrangement through homologous recombination.

The D antigen is clinically the most important because of its high immunogenicity and polymorphism. It has a prevalence rate of about 85% in Caucasians and about 95% in blacks (Daniels *et al.*, 2007; Poole and Daniels, 2007). The RHD negative phenotype is characterised by high molecular diversity between serologic and molecular methods. It accounts for the incidence of alloimmunization due to pregnancy or blood transfusion, despite the numerous red cell antigens (Delaney *et al.*, 2016; Egbor, Fellow and Knott,

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2012). Most red cell alloimmunization is attributed to antigens “D” “C”, “c”, “E”, “K”, and “Jk”a (Evers *et al*., 2016; Flegel *et al*., 2016). Alloimmunization in mothers is caused by pregnancy, blood transfusion, abortion, ectopic pregnancy, amniocentesis (iatrogenic) and foeto-maternal haemorrhage (Nour, ARamy and Ali, 2011).

The absence of the D antigen designates RH negative status on human erythrocytes; its molecular basis is divided into deletion and non-deletion types. Vast majority of Caucasians with the phenotype have a total deletion of the RHD gene. The non-deletion type occurs commonly in Africans, Japanese and other Asians (Huang, 2013). There are genomic alterations of three types that silence the D antigen expression in RH negative subjects who carry either a partial or completely intact RHD (Avent *et al.*, 2006; Flegel, 2011). The weak D (D^U) phenotype is associated with various forms of mutations that affect the quantity and quality of D antigen (Daniels, 2013; Singleton *et al.*, 2016). Most of these mutations are novel missense mutation that results in a single amino acid substitution that resides on either transmembrane domains or cytoplasmic portion of the protein. Partial D phenotype typifies a loss and/ or an alteration of one or more of D epitopes within the context of entire D protein. They often show weakened D expression making it difficult to differentiate between partial D and weak D (Denomme *et al*., 2005).

Due to the density and epitope expression of the D antigen on the red cell surface, the phenotypes commonly observed are normal D positive, weak D, partial D, DEL and D negative (Ye *et al*., 2014). Expression of weak D and partial D is attributed to variants of antigens and a large number of alleles of RHD gene (Rizzo *et al.*, 2012). The weak D

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phenotype is due to quantitative expression of the D antigen epitopes whiles that of the partial D is due to qualitative expression of antigenic epitopes of the D antigen.

The discovery of the Rh blood group system was an insight into the instances of the haemolytic disease of the foetus and new-born (HDFN). HDFN is perpetuated by foeto-maternal rhesus antigens incompatibility (Rouillac-Le Sciellour *et al.*, 2007). HDFN occurs as result of the immune-mediated destruction of red cells antigens of a foetus that are not usually found on maternal red cells ((Fasano, 2016). The common antibody produced is immunoglobulin G (IgG). The trans placental transfer of IgG antibodies attacks foetal RHD antigens leading to their destruction (Fasano, 2016). Other RH blood group antigens that can cause alloimmunization include antigen C, E, c and e (Shao *et al.*, 2010). Antibodies directed against epitopes of these antigens can cause alloimmunization through transfusion or pregnancy. The antibodies often encountered in Africa are anti-c, anti-C, anti-e and anti-D (Ngoma *et al.*, 2016).

1.2 PROBLEM STATEMENT

The highly immunogenic nature of the RHD antigen and the consequent risk of alloimmunization of RHD negative individuals, when exposed to RHD positive foetus or transfused with RHD positive blood is well documented (Ngoma *et al.*, 2016). Approximately 80% of RHD- negative individuals develop anti-D when exposed to D-positive blood or foetus (Wang *et al.*, 2010).

RHD antigen incompatibility has led to haemolytic disease of the foetus and new-born (HDFN) and haemolytic transfusion reaction. About 40% RHD negative women carry RHD negative children (Daniels *et al.*, 2007) and hence are not at risk of HDFN. The

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common variants of RHD antigens are difficult to detect serologically and are often mistyped as RHD negative by conventional serological assays (Gowland *et al.*, 2014). Some of these RHD variants have caused anti-D alloimmunization in transfused patients. D variants prevalence in Ghana has not be been established.

Though the inception of anti-D immunoglobulin prophylaxis has reduced the frequency HDFN, there is still alloimmunization to RHD antigens (Egbor, Fellow and Knott, 2012), especially in developing countries where there is, the lack of the anti-D immunoglobulin prophylaxis(Pal and Williams, 2015). Antenatal service provision remains an avenue for promoting maternal and neonatal health. It has being reported that low prenatal and maternal health service utilisation are among the factors contributing to adverse birth outcomes (Asundep *et al.*, 2013). Though there is relatively high patronage of antenatal services in Ghana (Gyebi, 2015), antenatal screening for alloantibodies in pregnant women, even if carried out, is at the tertiary hospitals. This approach has left out many potentially alloimmunised pregnant women (Egbor, Fellow and Knott, 2012), seeking antenatal at other levels of the health care system in Ghana.

Currently, data in Ghana shows that 48% of all under-five mortality occur within the first month of life (Gyebi, 2015)). RHD variants need to be investigated because of the risk of alloimmunization in patients especially pregnant women to prevent HDFN (Abdelkefi *et al.*, 2014; Sankaralingam *et al.*, 2016). Furthermore, anti-D immunoglobulin administration has theoretically exposed pregnant women to prions and other blood transmissible pathogens (Neovius *et al.*, 2016).

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1.3 SIGNIFICANCE OF STUDY

RHD negative women often require anti-D immunoglobulin prophylaxis especially during the third trimester of pregnancy or within 72 hours after delivery depending on the local policy. The use of anti-D immunoglobulin prophylaxis to prevent alloimmunization in RHD negative women has significantly decreased HDFN (Mcbain, Crowther and Middleton, 2015). In sub-Saharan Africa, management of RHD negative women has inherent challenges, which include the absence of policy on universal access to anti-D immunoglobulin, poor health infrastructure, lack of foeto-maternal testing facilities, unaffordability of anti-D immunoglobulin and suboptimal management and poor data on potentially sensitising events (Erhabor *et al.*, 2013). The majority of the potentially sensitising events of D antigen are silent (Mcbain, Crowther and Middleton, 2015). Routine anti-D immunoglobulin prophylaxis is only effective in non-sensitised RHD negative pregnant women. Approximately 40% of RHD negative women who carry RHD negative foetus will be given anti-D immunoglobin unnecessarily (Mcbain, Crowther and Middleton, 2015)). Furthermore, this prophylaxis cannot prevent all cases of sensitisation; some are either prior to or despite the administration of anti-D immunoglobulin.

Determining pregnant women with partial D or weak D phenotype may help in identifying pregnancies at risk of HDFN. Some weak D types (type 1, 2, 3 and 4.1) do not cause alloimmunization and pregnant women with such D types often do not require anti-D immunoglobulin prophylaxis (Hussein & Teruya, 2014). Those with weak D type 4.2, 11, 15 and 21 are the ones at risk of HDFN (Rizzo *et al.*, 2012). Pregnant women with common weak D types should be manage as RHD positive as this is clinically beneficial without increasing overall cost of unnecessary testing and routine

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antenatal care (Kacker *et al.*, 2015). Identifying pregnant women at risk of HDFN, that is the true RHD negative pregnant women, will eliminate the need for unnecessary administration of anti-D immunoglobulin prophylaxis. Screening for weak D and partial D will eliminate unnecessary administration of anti-D immunoglobulin prophylaxis in routine antenatal and postnatal care and enhances a cost-effective management of RHD negative mothers.

Molecular typing of pregnant women with weak D and partial D phenotypes may help prevent unnecessary anti-D immunoglobulin prophylaxis for individuals with weak D type 1, 2, 3, and 4. It will also prevent mistyping of partial D and weak D blood as D negative, thus conserving blood for the true RHD negatives (Shao *et al.*, 2010), with particular regards to transfusion cross-matching. RH incompatibility due to variant forms of the D and other RH antigens is still crucial for pregnancies and patients with chronic diseases who require frequent blood transfusion or organ transplant (Huang ., 2013).

1.4 AIM AND SPECIFIC OBJECTIVES

1.4.1 Aim

The aim of this study was to identify and classify rhesus D (RH1) variants among pregnant women, that are at risk of haemolytic disease of the foetus and newborn (HDFN).

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1.4.2 Specific Objectives

1. To screen for anti –D antibody in RHD negative pregnant women in order to determine those who are already sensitised against antigen D (RHD) and are at risk of HDFN.
2. To screen for molecular RHD status omitted by conventional serology which has the potential of causing alloimmunization in the pregnant women.
3. To determine the prevalence of RHD variants that can cause alloimmunization in the RHD negative pregnant women.

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CHAPTER TWO

LITERATURE REVIEW

2.1 THE RHESUS (RH) BLOOD GROUP SYSTEM

The rhesus (Rh) blood group system is the most important after the ABO blood group system. The Rh antigens form part of the largest human blood group system. More than 51 Rh antigens are described but the most important are the D, C, c, E and e antigens in transfusion practice (Quraishy and Sapatnekar, 2016). The Rh factor is a term used to describe the presence of the D antigen on the surface of erythrocytes. The D antigen was the first antigen that was identified belonging to the Rh blood group system (Huang., 2013).

The Rh factor and Rh antigens, at the population level, are highly polymorphic. The common potent Rh antigens reside on the RH polypeptides, whose expression is controlled by the RHD and RHCE genes, linked in tandem on chromosome one (1). The RHD produces the D antigen and RHCE produces the alleles Ce, ce, cE, CE compound antigens. Both RHD and RHCE genes, by postulation, are buried in the plasma membrane with 12 hydrophobic domains and, six hydrophilic and antigenic loops located on the outside of the membrane (Iwamoto., 2005). At the molecular level, about 300 allelic forms of the RHD and RHCE genes have been identified. This polymorphism is due to the various pattern of genomic diversification at the RH locus. The predominant mechanism of diversification at the RH locus is coding nucleotide changes and genomic rearrangement through homologous recombination. The RH antigens are erythroid specific at the transcriptional level (Flegel, 2011).

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However, RH antigen expression also involves genetic and physical interaction with a homologous partner, RH-associated glycoprotein (RHAG), whose gene is located on chromosome six (6) (Huang, 2013). The deletion or inactivation of one will affect the expression of the other.

RH deficient cells (RH null), is as a result of lack of expression RH antigens due to the mutation in the RH or RHAG loci. Rh null is a rare phenotype, characterised by the lack of expressions of Rh antigens on red cells. Rh proteins are necessary for the structure and function of the red cell membrane. Rh null phenotype can be associated with Rh deficiency syndrome which is characterised by stomatocytosis, spherocytosis, increased osmotic fragility, abnormal membrane phospholipids organisation, altered cell volume and altered ion transport system (Wafi et al., 2016).

Nevertheless, RH antigens are inherited in haplotypes that are usually designated by two common terminologies: the Fisher-Race terminology showing the combination of D, C, c, E, and e antigens, and the modified Weiner terminology which uses the letter R (D-positive) or r (D negative), with the presence of C/c and E/e antigens indicated by subscript to R or superscript to r.

The unusually large number of rhesus antigens are due to complex genetic basis (Flegel, 2007). The close association among the RH antigens is emphasized by the observation of weakened D antigen typing, influenced by the status of CE proteins present in a sample (Quraishy & Sapatnekar, 2016). The Rh factor importance clinically arises from

i. the nature of D antigen, a potent inducer of alloimmunisation
ii. power of anti-D alloantibodies to mount haemolytic damage and

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iii. Variability of the D antigen and high incidence of its negative phenotype in humans (Huang. 2013).

2.1.1 Molecular Basis of RH Alleles

Rh antigens are 12 hydrophobic membrane-spanning proteins that are uniquely palmitoylated at cysteine motifs on the endofacial loops (Huang ., 2013). The Rh locus is bigenic comprising of RHD and RHCE positioned in a tail to a tail orientation towards each other on chromosome 1 (Flegel, 2007). Rh polypeptides of D, C, c, E and e antigenic specificity migrate at ~ 30 kD position. These polypeptides have the same length of 417 amino acids residues with a molecular mass of 45.6 kD (Huang ., 2013). RH alleles may also encode low incidence antigens such as V and VS antigens that are expressed in 26-40% of Africans (Belsito, Magnussen and Napoli., 2017).

At the molecular level about 300 allelic forms of RHD and RHCE have been identified which reveals diverse patterns of genomic diversity at the RH locus. In the blood group antigen gene mutation database, most of these allelic variants carry amino acid changes that are different from the D and CcEe Rh polypeptides (Huang ., 2013). Majority of these Rh alleles harbour either single nucleotide polymorphisms (SNPs) or present as RHD/RHCE hybrid alleles. The tail-to-tail positioning might facilitate the large number of alleles of the two Rh genes (Flegel, 2011).

There is also hemizygosity of the RHD allele. In a Greek Cypriot population, there are RHD hemizygotes among RHD positives (Papasavva *et al.*, 2016). On that basis, a homozygous RHD negative mother with a hemizygous RHD positive partner has 21 % chance of bearing a homozygous RHD negative child (Papasavva *et al.*, 2016).

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2.2 RHD VARIANTS

As new RHD alleles are identified their characterization is necessary for the field of transfusion and foeto-maternal incompatibility (Filosa et al., 2016). Almost all people are either tested as RHD positive or RHD negative. The frequency of RHD phenotype (RHD positives) is about 85% in Caucasians, 95% in sub-Saharan Africa and 99.5% in East Asia (Daniels, 2013).

Although most people are often categorised as either RHD positive or negative, a grey area of profuse D variants do exist. These are generally referred to as weak D, partial D and DEL (Table 2.1). These D variants phenotype is roughly defined in terms that are of concern outside their clinical relevance. Blood grouping serology has been confronted with the issues of terminology and nomenclature related to these D variants. This confusion in nomenclature and terminology has led to an unjustified waste of D negative bloodstock (Daniels, 2013). When D antigen discrepancies arise, clinicians are challenged with designating the appropriated D antigen status. This provides the basis for administering the right D negative or positive blood product (Denomme and Flegel, 2008).

Red cell alloimmunisation is a serious threat in chronically transfused patients. The alloantibody production in these patients complicates red cell survival and safe transfusion practice (Belsito, Magnussen and Napoli, 2017). RH variants are difficult to serologically define. A DNA based typing will be assistive in matching donors and recipients especially for transfusion-dependent patients (Belsito, Magnussen and Napoli, 2017). The rate of alloimmunisation in transfused population ranges from 0.5 - 6.5%,whereas it is 8-36% in sickle cell patients (Belsito, Magnussen and Napoli, 2017).

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RHCE variant alleles are also found in 43% of sickle cell patients (Gaspardi *et al.*, 2016)

Table 2.1: D variants associated with alloantibody production (Adapted from (Daniels, 2013).

Partial D Category	Weak D Category
DII	Weak D type 1
DIII	Weak D type 2
DIVa	Weak D type 4
DIIIb	Weak D type 11
DV	Weak D type 15
DVI	Weak D type 21
DVII	Weak D type 57
DAR	DEL-5
DAU	DEL-ex8del
DBT	
DFL	
DFR	
DFV	
DHAR	
DHMI	
DMH	
DMI	
DNAK	
DNB	
DOL	
DWI	

The molecular mechanism behind this D variants include an amino acid substitution in the RHD protein as a result of few nucleotide changes in the RHD gene or gene

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conversion in which part of the RHD gene is replaced by RHCE (Daniels, 2013). In the 1950's and 60's, the concept of "D mosaic" was developed, in which normal D antigens is mosaic with all pieces present, but variants may have some pieces missing (Daniels, 2013). An individual who lacks parts of the D mosaic, and is immunised with normal D may produce antibody to the lacking parts. This antibody may be anti-D when tested serologically.

However, the concept of "D mosaic" was replaced by the concept of "D epitopes" in the 1980's. This development was coupled with the advancement of human monoclonal antibodies production. The concept of ''D^{U}'' was used when D antigen on red cells do not agglutinate with IgM Anti-D, but with IgG Anti-D in an antiglobulin test (Daniels, 2013). "D^{U}" was considered a quantitative variant of the D antigen-based number of antigens per red cell. With the advancement in monoclonal anti-D reagents, previously D^{U} red cells would be normal D antigens in routine testing. The consequence of this development led to the replacement of the term D^{U} with the term "weak D" in the early 1990's (Daniels, 2013). By this development, 30 epitopes of D variant cells were defined by using monoclonal antibodies against D variant cells (Scott *et al.*, 2014).

2.2.1 Partial D and Weak D Variants

Studies have shown that 1% of Europeans carry RHD variant allele expressing weak D or partial D (Wang *et al.*, 2010; Rizzo *et al.*, 2012). The antigenic arrangement of rhesus D antigen is in a mosaic fashion. A weak D phenotype is a consequence of quantitative expression of the D antigen epitope whiles that of the partial D is a qualitative antigenic expression of the antigens. Though subjects of D variants are serologically tested RHD negative, a small proportion still carries the RHD gene (Denomme *et al.*, 2005).

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Individuals lacking the mosaic epitopes can stimulate the production of alloantibodies when exposed to full D antigen(Pal and Williams, 2015). For instance, individual with partial D lack some of the epitopes of the D antigen. Such individuals are at risk of alloimmunization when exposed to blood with D antigens (Kulkarni *et al.*, 2008). The prevalence of partial D varies among different races and ethnic groups. In India, partial D prevalence is 0.15%(Kulkarni *et al.*, 2008). In Europe, it is about 0.2 to 1% of the subjects (Gaspardi *et al.*, 2016) RHD variants can occur up to 2.2% in some multi-ethnic studies (Wang *et al.*, 2010).

2.2.1.1 Weak D

Weak D is defined serologically as weaker or no reactivity of RBC with anti-D reagents when tested initially but substantial or moderate reactivity with the antihuman globulin addition (Virk and Sandler, 2015). The basis for weak D type's phenotypes is due to single nucleotide polymorphism (SNP) in RHD encoding amino acids changes within either the membrane-spanning domains or cytoplasmic loops of the protein (Arnoni *et al.*, 2014a). These changes could interfere with integrating of RHD proteins in membranes leading to reduced number of antigenic sites on the red cell (Arnoni *et al.*, 2014). Weak D presents all epitopes but shows quantitative epitope variability based on serology (Scott*et al.*, 2014).

It is estimated that 1% of the white (Caucasian) women have serologic weak D phenotype. In white populations, 95% of the weak D phenotypes are weak D type 1,2,3,4.0 and 4.1 (Virk and Sandler, 2015). Individuals with these weak D phenotypes are to be treated as RHD positives since they do not produce anti-D through blood transfusion or pregnancy. Some studies have identified anti- D autoantibodies in weak

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D types 1 and 2, by evaluating 121 weak D type 1 and 99 weak D type 2 (Flegel and Denomme, 2012). However, where there is no standard practice regarding the administering of anti-D prophylaxis; pregnant women with such phenotypes are administered with unnecessary anti-D prophylaxis. The American College of Pathologists considers testing for weak D as unnecessary for patients as well as pregnant women (Virk and Sandler, 2015).

In contrast, some weak D types are associated with alloimmunisation. These include the weak D type 4.2, 11, 15, 21, 57 and DAR. Classically, Weak D is usually tested serologically as D negative. In one particular case, a mother tested D negative serologically, but upon genotyping had RHD sequences present. This indicated RHD positivity, upon further confirmatory molecular typing. The variants identified were weak D type 1/dCcee and weak D type 2/dCcee (Papasavva *et al.*, 2016).

2.2.1.2 Partial D

Partial D is the term for qualitative expression of variants of D antigen, in which some or many epitopes are lacking, stimulating the production of D-like antibody. It is characterised by amino acid changes outside the membrane that modify or create new epitopes (Arnoni *et al.*, 2014). Testing partial D cells against D-like antibodies led to the categorisation of partial D. These initial categories were numbered from “DII to DVII” and were subdivided based on serological refinement. Furthermore, sequencing of genes of these antigens brought out a plethora of these partial D antigens. Some of which were later designated as “DBT”, “DFR” and “DHAR” (Arnoni *et al.*, 2014).

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2.2.2 Molecular Basis of D Polymorphism

The D antigen is a 417 amino acid moiety, with a tertiary conformation that is described in a 30 epitopes model (Denomme, 2004; Scott *et al.*, 2014). Putatively, 251 alleles of the RHD gene have been described (Banch *et al.*, 2014). The RHD antigen density ranges from 450-8804 amino acids (Filosa *et al.*, 2016). Individuals who have the D antigens on their red blood cells are RHD positive whiles those who do not have the D antigens are RHD negative. The RHD negative phenotype is attributed to deletion of the RHD gene in most white(Caucasian) populations (Kumar and Regan, 2005; Jemni Yacoub *et al.*, 2013). On the contrary, in Africans and Asians, RHD negative phenotype can be due to the qualitative and quantitative expression of the D antigen.

Weak D is basically due to a SNP in the transmembrane domains or intracellular regions resulting in reduced quantitative expression of D antigen (Hussein & Teruya., 2014). Partial D is due to a mutation in extracellular regions and exchange mutation between RHD Exons and RHCE Exons cumulating in altered or new epitopes of D antigen. It has been demonstrated that 90% of D- negative individuals have a deletion of the RHD gene while 9.2% pose RHD polymorphisms(Arnoni *et al.*, 2014)). About 50 molecular weak D types are documented. In the white populations, 90% of the weak D types are type 1, 2 and 3. Among the weak D types, type 1, 2, 3, and 4.1 do not cause alloimmunization when exposed to the D antigen. Weak D types 4.0, DAR(4.2), 11 and 15 have been associated with alloimmunization in transfused patients (Hussein & Teruya., 2014). On the other hand, all partial D-types have been associated with sensitization to the D antigen.

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2.3 PSEUDO RHD GENE (RHDΨ)

RHDΨ is characterised by insertion of 37 base pairs (bp) in exon 4 (Singleton *et al.*, 2016). The insert is sequence duplication across the boundary of intron 3 and exon 4 and introduces a stop codon at position 210. The duplication of the 37 bp transcends the last 19 nucleotides of intron 3 and first 18 nucleotides of exon 4, together with four nonsense mutation in exon 5 and a nonsense mutation in exon 6 (Ziza *et al.*, 2017). Nevertheless, RHDΨ also contains another stop codon at exon 6. The pseudogene is also associated with RHCE*ce alleles.

In tested RHD negative black Africans, 67% had both regions of RHD gene, 15% had RHD exon 10 and 18% lacks RHD. The RHDΨ in RHD negative African American is 24%, whilst 17% in South Africans. The frequency of the RHDΨ gene is 0.0714 in South African population (Reid *et al.*, 2014; Singleton *et al.*, 2016).

In other studies among 203 RHD negative blood donors, the prevalence of RHDΨ was 3.4% (Szulman *et al.*, 2012). Nonetheless, the predominant Caucasian nature of donors could be accountable for the lower prevalence as compared to studies among black populations. Individuals of African descent are heterozygous, hemizygous or heterozygous for the RHDΨ gene (Singleton *et al.*, 2016)

2.4 SEROLOGIC TYPING OF RHD VARIANTS

Serologic testing is still the main stage in transfusion science testing, if the test is well characterised and sensitive enough to find and identify most relevant clinical alloantibodies that are beneficial to patient situation(Jeremiah *et al.*, 2011; Boggione *et al.*, 2014). However, serologic testing is limited in peculiar patients and clinical

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situations. An exhibition of this is in patients with substantial alloantibodies such as passive maternal antibodies in neonate (El Wafi *et al.*, 2017), likewise patients with antigens or antibodies for which testing antibodies or antisera are not available such as partial or variant antigens, rare antigens, and in patients with antigen zygosity that need to be determined (Elkins *et al*., 2013).

Molecular DNA-based testing offers an alternative (Fasano & Chou., 2016). Although molecular typing of RHD variants resolves the discrepancies, serologic typing using an array of monoclonal antibodies is useful in resource-limited laboratories (He *et al.*, 2015). Based on this monoclonal antibody serology, 97% of discrepant RHD cases were rectified using 12 monoclonal antibodies (Kulkarni *et al*., 2013). Other studies concluded that partial D and weak D cannot be distinguished by serology, but hint that the probable RHD variants can be confirmed by molecular typing (Hussein and Teruya, 2014). The presence or absence of anti-D does not differentiate serologic weak D and partial D phenotypes. Furthermore, a weak RBC antigen cannot be detected by serology and sensitivity of serology reagents varies between manufacturers.(Wagner, Frohmajer and Flegel, 2001) demonstrated that in weak D types typing, using IgM and IgG antibodies resulted in negative results. (Orzińska *et al.*, 2013; Arnoni *et al.*, 2014).

A survey by the College of American Pathologists (CAP) stated that there is no codified practice for interpreting RHD type when serologic weak D is detected (Sandler and Gottschall, 2012; Sandler *et al.*, 2014; Sandler, Chen and Flegel, 2017). The majority of laboratories in that study routinely did not use indirect antiglobulin test, avoiding the detection of weak D phenotype. Consequently, 60% of pregnant women in the USA having serologic weak D phenotype, received unnecessary anti-D immunoglobulin

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prophylaxis because they will deliver RHD positive babies (Sandler *et al.*, 2015). Annually 13,360 women with serologic weak D can safely be manage as RHD positive without administering anti-D immunoglobulin prophylaxis in the (Scheffer *et al.*, 2013; De Haas *et al.*, 2017)

2.5 ANTIBODIES TO BLOOD GROUP ANTIGENS

The discovery of universally, naturally occurring antibodies in blood plasma led to the discovery of ABO blood group system some 100 years ago (Poole and Daniels, 2007). Antibodies to blood group antigens are naturally occurring or from exposure to the environmental antigens that are similar to RBC antigens. Other antibodies are induced as a way of the immune response after exposure to antigens on red cells through transfusion or pregnancy. Antibodies to foreign antigens are termed alloantibodies whilst those against self-antigens are autoantibodies(Urbaniak, 2006). The clinical significance of these antibodies is their ability to mediate destruction of RBCs. These antibodies are of importance to transfusion medicine because of their ability to cause an adverse transfusion reaction and their implication in HDFN.

The intrinsic ability of an antigen to induce the immune response of antibody production depends on the nature of antigens. The immune response to Rh antigens is T-cell dependent, which leads to classical primary and secondary humoral immune responses. The humoral immune response starts with IgM but quickly maturing to IgG class. In the peculiar case of RhD alloimmunisation in antenatal cases that can result in haemolytic disease of the foetus, the antibody is usually of the IgG class (Quraishy & Sapatnekar., 2016). However, the frequency of alloantibodies in transfusion recipients is greater than that associated with pregnancies (Urbaniak. 2006). RhD negative

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alloimmunisation is found in 10% of antenatal women. The prevalent antibody specificity causing the alloimmunisation is the anti-D antibody (Sankaralingam *et al.*, 2016).

2.5.1 ABO System Antibodies

ABO antibodies are naturally occurring and the most encountered antibody class is IgM. They bind complement, with a significant quick clearance of red cells, after incompatible red cell transfusion. Alloantibodies related to ABO blood group system are anti-A1 and anti-H. Anti-A1 reacts with 80% of blood group A cells (Hamilton, 2009). This antibody has rarely been reported to cause red cell clearance. It is managed by selecting group O or group A crossmatch compatible blood donor units, regardless of A1 status.

Anti- H antibody though not related to the ABO blood system is related to the ABO antigens. The H antigen on red cells is converted to A or B antigens by the ABO reactive enzymatic transferases. Group O without the H antigen conversion, have the highest amount of H antigens. Anti-H is a cold antibody made frequently by group A1 or B (or AB) (Hamilton, 2009).

ABO HDFN occurs in infants of group A or B born to group O mothers. IgG anti-A or anti-B is common in group O mothers than in group A or B mothers. It is an important cause of hyperbilirubinemia. ABO incompatibility occurs in 0.33 to 2% of neonates, and about 15% of group A or B offspring of group O mother are at risk (Deng et al., 2008). ABO HDFN is often benign, characterised by hyperbilirubinemia, moderate anaemia with cases of reticulocytosis and spherocytosis.

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2.5.2 RH Antibodies

Most RH antibodies are an IgG class with few IgM. Most do not bind complement. The anti-D is the most common of all the RH antibodies. The incidence of alloantibodies in multi-transfused patients against RBCs was 84.76% (Younesi *et al.*, 2016). These patients had alloantibodies. The common antibodies were anti-K, anti-E and anti-D

Rh related antibodies are stimulated by five main antigens out of the 43 designated Rh antigens, and the five main antibodies produced are the anti-D, anti-C, anti-E, anti-c and anti-e (Hamilton., 2009). They are found together in circulation, because of inheritance of common Rh haplotypes. Individuals who are e- (lack e antigen) are commonly c- (lack c antigen) too, and therefore anti-e and anti- c are commonly found in alloimmunised patients. Likewise, C- individuals are usually E-, producing anti-C and anti-E, respectively. RHD negative phenotype also has linked antigens. It is common to find D- subjects who are also C- and E- (Hamilton., 2009).

Regardless of the antibody type, the pathogenesis of cell-mediated haemolysis is the same. Once antibodies enter the foetal circulation, they can coat the foetal red cells, if positive for the corresponding antigen and initiate destruction or removal from circulation by macrophages of the foetal spleen (Pal and Williams, 2015).

Antenatal screening programmes detect clinically significant antibodies in 0.24-1% of pregnant women. Although the D antigen is the most frequent cause of severe HDFN, there are other antigens that are also implicated (Pal and Williams, 2015; Nguyen and Bhargava, 2016).

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2.6 MATERNAL ALLOIMMUNISATION

Alloimmunisation is one of the causes of perinatal mortality and morbidity. It affects 1-2% of all pregnancies and approximately 50% of Rhesus immunised pregnancies (Nour *et al.*, 2011). Maternal alloimmunisation occurs when a woman's immune system is sensitised by foreign surface antigens, stimulating the production of immunoglobulins, usually IgG antibodies.

Among the 51 antigens capable of causing maternal alloimmunisation and foetal haemolytic disease, the rhesus blood group is the commonest. The Rh blood group antigens are carried by a series of at least three homologous but distinct membrane-associated proteins. Two of these proteins have immunologically distinguishable isoforms designated C, c and E, e. The principal protein D has no immunological detectable isoform, so its presence or absence in the genome determines the genetic basis of the polymorphisms associated with Rh positivity and Rh negativity(Din and Ali, 2011; Nour *et al.*, 2011). In the 1960 Stem found out that sensitization to Rh-positive blood could be prevented by administering anti-D immunoglobulin. Anti-D gamma immunoglobulin contains immunoglobulin IgG, manufactured from a pooled source of plasma males and post-menopausal women (Mcbain, Crowther and Middletonl., 2015). The donors must be RhD negative and can be immunised to produce anti-D or to increase anti-D titre (Ziza *et al.*, 2017).

When anti-D immunoglobulin became available in the early 1970s, death from haemolytic disease dramatically reduced, with the post-partum administration. The administration effectively protected against rhesus alloimmunisation when properly used. The benefits were seen when anti-D was given within 72 hours after birth

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regardless of mother's ABO blood group status (Mcbain, Crowther and Middleton, 2015). In an RHD negative mother who does not receive anti-D immunoglobulin prophylaxis after delivery the sensitisation is 12- 16% compared to 0.8 - 1.5 % in those who receive the prophylaxis (Johnson *et al*., 2017).

Nevertheless, sensitising events may occur during pregnancy or blood transfusion and postpartum anti-D might not prevent rhesus alloimmunisation which occurs in the antenatal period (Ahrens *et al*., 2007). The most common alloimmunising event is during delivery but those that occur during pregnancy can be due to unrecognised foeto-maternal haemorrhage (FMH) that increases with maternal age (Johnson *et al*., 2017). The high rate of alloimmunisation is correlated with failure to attend antenatal clinics, and improper administration of prophylaxis or non- availability of prophylaxis (Ziza *et al*., 2017). However, RHD alloimmunisation risk has been reduced from around 13% to 1-2% by the administering post-natal anti-D prophylaxis (Neovius *et al*., 2016). This postnatal ant-D prophylaxis is still the routine practice in many countries including Ghana.

2.7 FOETO-MATERNAL HAEMORRHAGE (FMH)

Foeto-maternal haemorrhage (FMH) incidence increases with age of gestation. It is connected with factors such as trauma, multiple gestations and other invasive procedures (Virk & Sandler., 2015). .

In the United States, RHD immunoglobulin prophylaxis is given to pregnant females who have not form antibodies against the D antigen (Tiblad *et al*., 2013). It is a standard policy for RHD negative women and girls who carry an RHD positive foetus or the

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partner is RHD positive or rectified foetal RHD status. The policy involves a laboratory test of detecting FMH, quantifying the FMH, estimation of the volume of FMH, and using the volume to calculate the dose of RHD immunoglobulin. The gold standard of the procedure involves: rosette foetal RBC screening, which is a sensitive test that detects the presence of small amount of foetal RHD positive RBCs in the midst of large number RHD negative maternal RBCs. This assay is less sensitive in a foetus with weak D type's phenotype (Pal and Williams, 2015).

A quantitative estimation of the volume of FMH is by acid elution assay (Sandler and Gottschall, 2012). This assay is based on the continuous resistance of foetal haemoglobin (HbF) to acid elution compared to the adult haemoglobin (HbA). Briefly, a maternal blood specimen is fixed on a slide, exposed to buffered citric acid, and stained with erythrosine B and fast green. The HbF cells stained dark pink due to their resistance after elution in citric acid whilst the HbA cells appear as ‘’ghost cells’’. A minimum of 2000 foetal cells are counted microscopically and a percentage of the foetal cells in the maternal specimen is determined against the maternal ghost cells on the background (Virk & Sandler., 2015).

The volume of FMH is estimated conventionally by using the formula proposed by the American Association of Blood and Blood Banks (AABB) technical manual (Kumpel *et al.*, 2014). This formula assumes that the maternal blood volume is 5000 ml. Alternatively, the maternal blood volume can be calculated using height and weight. FMH volume (ml of whole blood) = maternal blood volume × percentage of foetal RBC s (result of the acid elution test). The dose of the RHD immunoglobulin required to prevent alloimminisation is calculated with known the volume of FMH:

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The number of phials = volume of FMH/30 ml.

In the United kingdom 100 µg dosage anti- D immunoglobulin is recommended to prevent alloimmunisation by up to 4-5ml of RHD positive foetal RBCs – preventing about 99% of FMH (Gregory, 2013). In America, the recommended dose is 250 -300 ug. However, 0.2% of women who have had FMH larger than 15 ml RBCs, with no adequate dose of anti-D immunoglobulin are at risk of sensitisation (Gregory, 2013)

2.8 HAEMOLYTIC DISEASE OF FOETUS AND NEWBORN (HDFN)

Haemolytic disease of the foetus and new-born (HDFN) is as a result of alloimmunization to paternally originating antigens on erythrocytes of the foetus or new-born (Stowell *et al.*, 2013). The first recorded incidence of HDFN was in 1609 by a French midwife (Kumar & Regan., 2005). The disease is triggered by vulnerability to erythrocytes antigenic stimulation through pregnancy or transfusion. For RHD immunisation to occur in a pregnant woman there must be certain conditions: The foetus must have RHD positive erythrocytes and mother must have RHD negative erythrocytes. A sufficient number of foetal erythrocytes must cross the placental membrane and gain access to maternal blood circulation. The mother must have the immunogenic capability of producing antibodies directed against the D antigen. The antibodies produced must be capable of crossing foeto-maternal membrane and causing the destruction of foetal erythrocytes (Neal, 2001).

Introduction of anti-D immunoglobulin prophylaxis regime, has significantly reduced the incidence of HDFN (Wong, 2013; Banch Clausen, 2014; Fasano, 2016a). Besides, the routine antenatal anti-D prophylaxis adopted by many nations have contributed significantly to this achievement (Fasano, 2016). On the contrary, there are some

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failures of anti-D immune prophylaxis due to FMH or insufficient amount of IgG in antenatal and postnatal subjects (Koelewijn *et al.*, 2009). In instances where there is no FMH, it is advisable to administer anti-D immune prophylaxis within 72 hours postnatal, and special circumstances within 13 days (Lubusky., 2010). The highest period of alloimmunization to D antigen occurs during the third trimester and labour (Clausen, Damkjær and Dziegiel, 2014). However, sensitisation can happen in both first and second trimesters.

In Europe, 60% of RHD negative pregnant women carry RHD positive foetus (Jones *et al.*, 2004) and 40% carry RHD negative foetus. The latter group do not need any form of prophylaxis since they are not at risk of HDFN (Clausen, Damkjær and Dziegiel, 2014). Hence, administering the immunoglobulin prophylaxis to all RHD negative women (Fasano, 2016) without prior knowledge of foetal RHD phenotype is a drain on the overburdened health resources in resourced constrained settings.

2.9 FOETAL RHD TYPING OF RHD NEGATIVE PREGNANT WOMEN

Lo and co-workers (Lo *et al.*, 1997) discovered that, foetal cell-free DNA was found in maternal plasma. After 8 weeks of gestation, plasma samples of RHD negative women were used to diagnose the presence of foetal RHD gene (Rouillac-Le Sciellour *et al.*, 2007). This protocol replaced the invasive determination of foetal RHD phenotype through amniocentesis. The benefit of this protocol eliminates invasive amniocentesis, which in itself can lead to sensitization. The basis for non-invasive RHD genotyping includes limited availability of anti-D and rare but risk of harmful effects such as virus transmission (Neovius *et al.*, 2016). In addition, it is unethical to expose 40% of women

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who carry RHD positive foetus to blood products that they do not need (Neovius *et al.*, 2015).

As a screening assay, the sensitivity is approximate >99.3% even in the first trimester of gestation (Clausen, Damkjær and Dziegiel, 2014). It has been demonstrated that 3% of pregnant women, 12% of pregnant women and 46% of pregnant women in first, second, and third trimesters respectively, have about 0.01 ml of foetal DNA found in maternal plasma (Kumar & Regan., 2005). A cell-free foetal DNA emanates from apoptotic micro vesicles. Apoptotic micro vesicles are part of the placental syncytiotrophoblast layer, that contains cytotrophoblast-derived foetal nuclei (Clausen, Damkjær and Dziegiel, 2014). This cell free DNA technique is increasingly being used for screening and diagnosis of genetic disorders prenatally (Wikman et al., 2012).

In determining the RHD status of a foetus by the polymerase chain reaction (PCR), absence -of exons 4, 5 and 10 signals confirms foetal RHD negative status. The foetus is RHD positive when two positive signals are obtained from each RHD exon together with two or more positive signals from other exons (Papasavva *et al.*, 2016) Non-invasive foetal RHD typing in routine practice is used to guide targeted antenatal anti-D immunoglobulin prophylaxis (Clausen, Damkjær and Dziegiel, 2014). A RHD negative pregnant woman is currently managed optimally, by determining the foetal RHD status-using cell-free foetal DNA in maternal plasma. This is being regularised in a number of countries around the world (Bills & Soothill., 2014). Foetal RHD genotyping has reduced the total use of anti-D by 25.3% (Papasavva *et al.*, 2016)

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CHAPTER THREE

METHODS

3.1 STUDY DESIGN

The research was a descriptive cross-sectional study.

3.2 STUDY SITES

Three health facilities, Ussher and Mamprobi polyclinics and 37 Military Hospital, were used for the study. Both Ussher and Mamprobi polyclinics are government owned polyclinics in James town and Mamprobi, respectively, in the Accra Metropolitan District of the Greater Accra region. Ussher polyclinic is located directly east of the Korle Lagoon whilst Mamprobi polyclinic is located south of Laterbiokorshie, northeast of old Dansoman and north of Chorkor. Both polyclinics offer antenatal services on a daily basis.

The 37 Military Hospital is a specialist hospital located in Accra, on the main road between the Kotoka International Airport and central Accra. It is the largest military health facility in the republic of Ghana. The hospital offers antenatal services to the military and the public on a daily basis.

3.3 SUBJECTS

The study population were all pregnant women attending antenatal care at the three study health facilities.

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3.3.1 Inclusion Criteria

All RHD negative, confirmed pregnant women at any period of gestation, attending antenatal care and who consented, were included.

3.3.2 Exclusion Criteria

All RHD positive pregnant women were excluded. All RHD negative pregnant women who had previously received anti-D immunoglobulin prophylaxis were also excluded from the study.

3.4 SAMPLE SIZE

The sample size was calculated using a simple formula. Using a confidence interval of 95% and a margin of error of 5%, the calculation was as follows:

$$n = \frac{[Z]^2 P(1-P)}{d^2}$$

$$= \frac{[1.96]^2 x\, 0.5(1-0.5)}{0.025^2}$$

= 384 participants

Where:

n is estimated minimum sample size

d is the precision.

z is the critical z score based on the desired level of significance.

P is expected prevalence of RHD negative variants among pregnant women in Ghana.

This study took taking P= 0.5 because there is no established prevalence of RHD negative variants in the Ghanaian population.

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However, 110 pregnant women consented to partake in the study.

3.5 BLOOD SAMPLES

Venous blood (5 ml) samples were drawn from each pregnant woman into anticoagulated EDTA tubes. Blood samples were collected on a daily basis at the three study sites. Only blood specimen with RHD negative phenotype tested through hemagglutination reaction was used.

3.6 CONFIRMATION SEROLOGICAL TYPING OF RHD STATUS

All blood samples were retested with monoclonal antibodies for RHD status for laboratory confirmation of the RHD status. RHD phenotypes were determined by haemagglutination with commercial anti-D IgM and anti-D IgG monoclonal antibodies (Rapid Labs Limited, United Kingdom) according to manufacturer's instruction. Samples that had a weak D expression were confirmed by indirect Antiglobulin tests.

3.7 CONFIRMATION OF WEAK D EXPRESSION

Weak D expression of the RHD negative blood sample was determined by anti-human globulin test (AHG anti-IgG) using an Ortho Biovue column agglutination technology. This method detects antibodies (IgG) in pregnant women that can result in HDFN (Dajak *et al.*, 2014). The subject's serum was extracted from the blood and incubated with RBCs of known antigenicity to detect antibodies against RBC, present but unbound in the subject's serum.

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3.8 ANTIBODY SCREENING

Antibody screening was performed using Ortho Biovue column agglutination technology (AHG polyspecific, C3d). All RHD negative blood samples were centrifuged at 56 rpm for 5 minutes to separate plasma from cellular components. The separated plasma was stored at -20°C until further testing. A commercially available three-cell antigen panel using gel cards (Ortho-clinical Diagnostics Inc., USA) was used to detect antibodies in the serum by reacting them with red cells using low ionic strength saline (LISS) in Coomb's gel cards. The gel cards used contained anti-IgG C3d polyspecific antibodies.

Briefly, the foil seal was removed from an individual micro tube of gel cards. The reagent red cells carefully mixed (group O positive and group O negative red cells). A 50 µl of reagent cells were added into each of the Microtubes. Fifty (50) microlitre of patient sera was added into each Microtube. The Control was prepared by adding anti-D reagent into the Microtube. Each Microtube was incubated at 37ºC for 10 minutes. The gel cards were spun at 56 RPM for 5 minutes and the results read.

3.9 DNA EXTRACTION

DNA was extracted from each RHD negative blood sample using QIAamp mini protocol (QIAamp DNA mini protocol USA) following the manufacturer's instruction. Briefly, 20 µl of QIAGEN protease was pipetted into a 1.5 ml micro centrifuge tubes. Then 200 µl of each blood sample was added to the micro centrifuge tubes followed by 200 µl of Qiagen Buffer AL and vortexed for 20s. The mixtures were incubated at 56°C for 10 minutes and spun to remove drops from inside the lids. Then 200 µl of ethanol (98%) was added to each sample, vortexed for 20s and centrifuged to remove drops

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from inside the lids. The mixtures were transferred into QIAamp mini spin columns fixed in 2 ml collection tubes, without wetting the rims, the caps closed, and centrifuged at 800 rpm for I minute. Then the QIAamp mini columns were placed in clean 2 ml collection tubes and the tubes containing the filtrate were discarded. The QIAamp spin columns were carefully opened and 500 µl of buffer AWI added to each column without wetting the rims, the caps closed and centrifuged at 8000 rpm for 1 min. Next, the QIAamp mini columns were placed in clean 2 ml collection's tube and the tube containing the filtrate discarded. Next, the QIAamp mini spin columns were carefully opened and 500 µl of buffer AW2 was added, without wetting the rims, the caps closed and centrifuged at 14000rpm for 3 minutes. The QIAamp mini spin columns were placed in new clean 2 ml collection tubes and centrifuged at full speed for 1 minute. This was followed by placing them in new clean 1.5 ml micro centrifuge tubes. The QIAamp mini spin column tubes were carefully opened and 200 µl of buffer AE was added, incubated at 15-25°C for 1 minute and centrifuged at 8000rpm for 1 minute.

The 200 µl of whole blood yielded approximately 12 µg of DNA. The DNA quality was checked by agarose gel electrophoresis (section 3.11).

3.10 MOLECULAR TYPING OF RHD VARIANTS

DNA amplification was carried out by multiplex PCR to amplify RHD and RHCE genes in the each RHD negative blood sample. Sequence specific primers were used to flank the exons harbouring the mutations (Fig. 3.1). The exons 7 and 10 were amplified using RHD specific primers as described previously (Bennett *et al*., 1993; Ouchari *et al*., 2013). Briefly, the forward primer A1 (5′-TGTGTTGTAACCGAGT-3′) and reverse primer A2 (5′-ACATGCCATTGCCG-3′), and forward primer A3 (5′-

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TAAGCAAAAGCATCCAA-3′) and reverse primer A4 (5′-ATGGTGAGATTCTCCT-3′) were used. The A1, A2, and A3 have nucleotides sequences that are identical to both RHCcEe and RHD cDNA, whiles the A4 primer is specific to the D sequence.

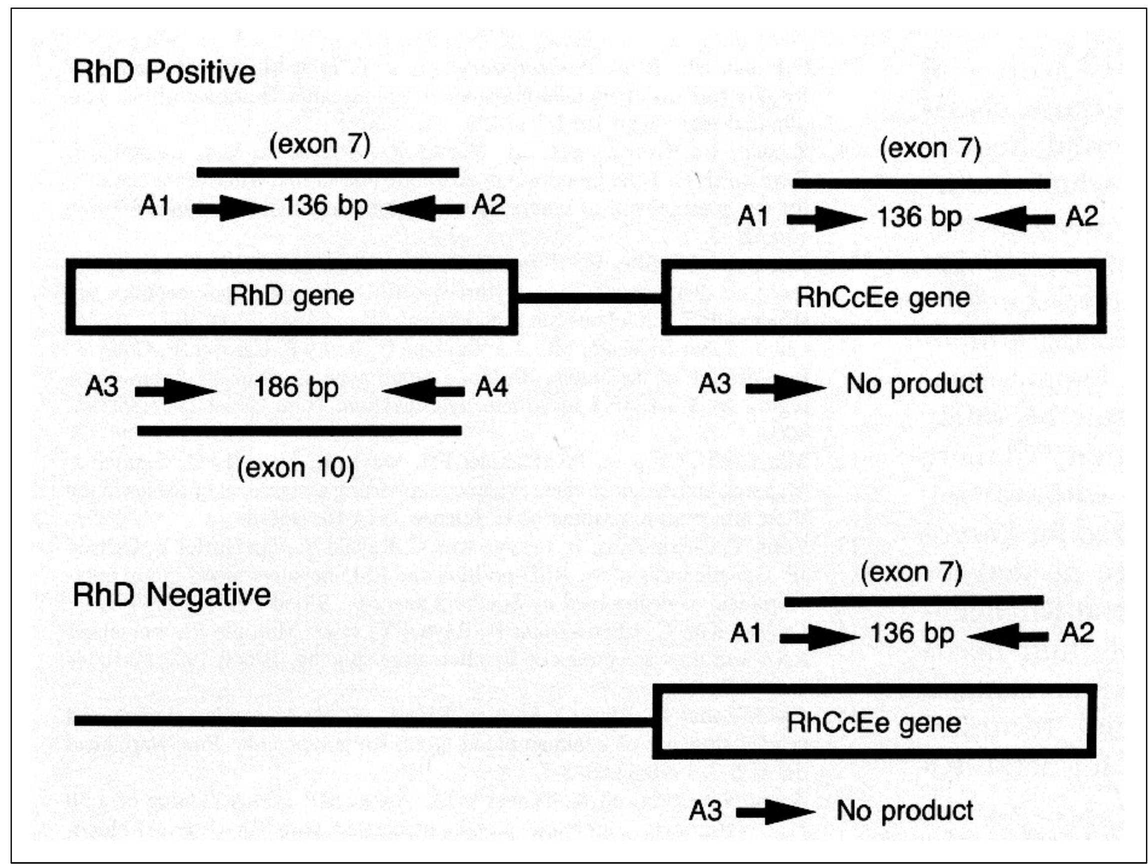

Figure 3.1: Principle of the amplification of the RhD-Specific region and the combined RhD and RhCcEe regions of the rhesus gene locus. Only the 136-bp product will be amplified from RhD-negative chromosomes, whereas both the 136-bp and 186-bp products will be amplified from RhD-positive chromosomes. A1, A2, A3, and A4 are the primers used for PCR. (Source: (Bennett *et al.*, 1993)

A total PCR reaction volume of 15 µl was setup. Each reaction volume comprised of 7.5 µl of x2 One *Taq* buffer (New England Biolabs, UK), 0.4 ul of each 10 µM primer

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and 3 uμl of DNA. The PCR reaction conditions were an initial melt at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 45°C for 1 minute, and extension at 68°C for 2 minutes, then a final extension at 68°C for 10 minutes. Each PCR reaction was mixed thoroughly. The PCR reaction was carried out in a SEEAMP SCE1000 thermal cycler (Seegene Inc., Korea). A RHD positive sample was used as an internal control.

3.11 MULTIPLEX PCR ANALYSIS

PCR analysis to reveal partial D and some weak D alleles was performed by amplifying RHD exons 3 through 7, and 9 with RHD sequence specific primers, as described by (Maaskant-van Wijk, et al., 1998) [Table 3.1]. The multiplex PCR conditions used in this study were slightly modified from those described by Maaskant van Wijk et al. (1998). A total PCR reaction volume of 15 μl was setup. Each reaction volume comprised of 7.5 ul of x2 One *Taq* buffer (New England Biolabs, UK), primer sets at different concentrations 0.4 -0.6 ul of each 10 μM primer, and 3 ul of DNA. A pair of nucleotides was used to generate a 434 bp PCR fragment from the human growth hormone gene (Table 3.1) which was included as an internal control to avoid false negative results.

Amplification was performed in a SEEAMP SCE1000 thermal cycler (Seegene Inc., Korea). After an initial denaturation at 94°C for 2 min, a total of 32 cycles were carried out using the following sequence: denaturation at 94°C for 3 min, primer annealing at 60°C for 1 minute, polymerization at 68°C for 45 s, and one cycle at 68°C for 5 min to complete extension.

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Table 3.1: Primers for multiplex PCR.

Primer	**Sequence**	**RhD exon**	**Size (bp)**
R364/F	TCGGTGCTGATCTCAGTGGA	3	111
R474M/R	ACTGATGACCATCCTCATGT		
R496/F	CACATGAACATGATGCACA	4	126
R621/R	CAAACTGGGTATCGTTGCTG		
R648/F	GTGGATGTTCTGGCCAAGTT	5	157
Rex5AD2/R	cacCTTGCTGATCTTACC		
R898M/F	GTGGCTGGGCTGATCTACG	6	57
Rex6AD3/R	tgtctagtttcttacCGGCAAGT		
R973/F	AGCTCCATCATGGGCTACAA	7	96
R1068/R	ATTGCCGGCTCCGACGGTATC		
Rex9SD2/F	aacagGTTTGCTCCTAAATATT	9	71
R1219M/R	AAACTTGGTCATCAAAATATTTAACCT		
HGH-F	TGCCTTCCCAACCATTCCCTTA	HGH gene	434
HGH-R	CCACTCACGGATTTCTGTTGTGTTTC		

3.12 AGAROSE GEL ELECTROPHORESIS

Following the PCR, 10 µl of each PCR product was added to 2 µl of 6 x bromophenol blue loading dye and electrophoresed in 2% or 4% (for the multiplex PCR) agarose gels stained with 0.5 g/ml ethidium bromide. The gels were prepared and run in 1x TAE buffer at 120V for 20-30 mins and were observed and photographed over a UV trans illuminator at short wavelength using a Kodak EDAS 290 gel documentation system. The sizes of the PCR products were estimated by comparing with the mobility of a standard 100 bp DNA ladder. Expected amplicon sizes were 136 bp for exon 7 and 186 bp for exon 10. The sizes of the PCR products were estimated by comparing with the mobility of a standard 100 bp DNA ladder.

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3.13 ETHICAL CONSIDERATIONS

Approval for the study was obtained from the Ethics and Protocol Review Committee of the School of Biomedical and Allied Health Sciences, University of Ghana. Approval was obtained from the Greater Accra Regional Health Directorate for samples to be taken at Ussher and Mamprobi polyclinics. The participants were introduced to the aim of the research as part of the process of seeking their consent. Participants signed and were given a copy of the informed consent form. For participants who could not read and write, the aim of the study was explained in the language they understood and a verbal consent was obtained.

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CHAPTER FOUR

RESULTS

4.1 CONFIRMATION OF SEROLOGICAL TYPING OF RHD STATUS AND WEAK D EXPRESSION

All the 110 samples were confirmed as RHD negative by serology using monoclonal antibodies, except for 2 samples that had weak D expression. The two samples were confirmed with indirect antiglobulin test as RHD negative.

4.2 ANTIBODY SCREENING BY GEL METHOD

Eight (7.3%) out of the 110 RHD negative samples showed the presence of red cell antibodies in the serum.

4.3 PCR SCREENING FOR RHD STATUS

All 110 RHD negative samples were screened for the presence of exon 7 and/or 10 of the RHD gene. Thirty-four (34) samples (30%) had both exon 7 and 10, depicting an RHD positive phenotype, 67 samples (60%) had only exon 7 amplified, denoting RHD negative phenotype and 9 samples (8.2%), had neither exon 7 nor 10.

Samples that were RhD-negative (exon 7) showed only the amplified 136-bp PCR product (Fig. 4.1). On the other hand, samples that were RhD-positive showed both the amplified 136-bp and 186-bp PCR products (Fig. 4.1), for exon 7 and exon 10, respectively.

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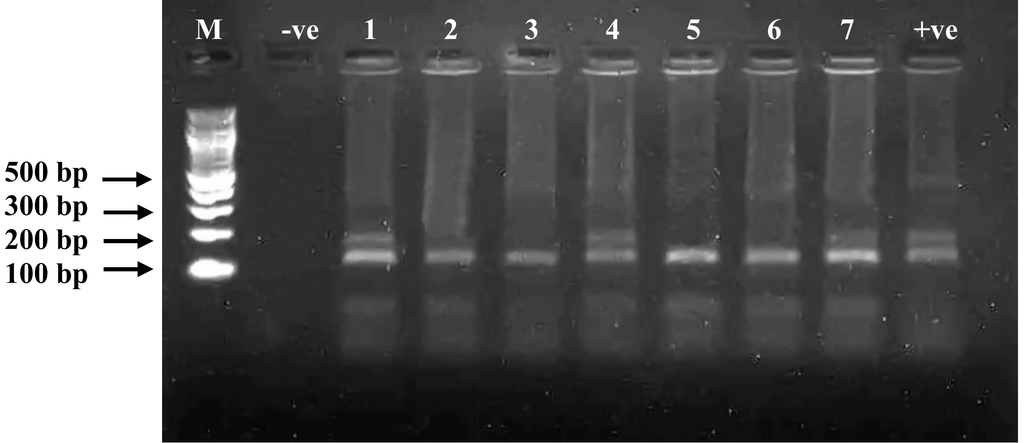

Fig. 4.1: Ethidium bromide stained with 2% agarose gel electrophoregram of amplified regions of both the RhCcEe and RhD genes (136 bp) and a region specific to the RhD gene (186 bp). In RhD-negative samples (2, 3, 5 and 6) there is no amplification of the 186-bp RhD-specific region. In RHD-positive samples (1, 4, and 7) amplification of the 186-bp RhD-specific region shows that these sample are RhD-positive.

Lane M = 100 bp ladder; Lane -ve = negative controls; Lane +ve = positive control

4.4 COMPARISON OF MOLECULAR RHD STATUS AND SEROLOGICAL RHD STATUS

Discrepancies were observed between the serology and PCR tests (Fig. 4.2). Only 67 (60.9%) of the 110 serologicallyconfirmed RHD-negatives were RHD-negative by PCR. In addition, 9 (8.2%) samples out of these 110 RHD-negative samples could not be typed by PCR(nether exons 7 nor 10 was present) .

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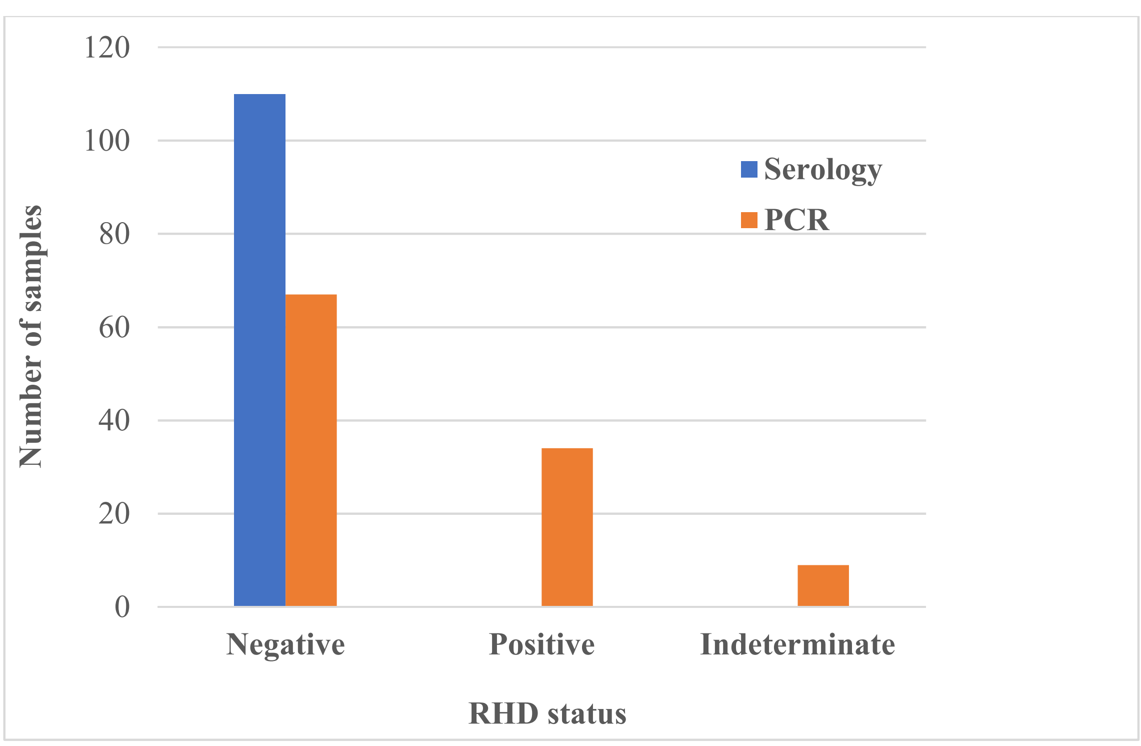

Fig. 4.2: Comparison of molecular RHD status and serological RHD status.

4.5 RED CELL ANTIBODY AMONG RHD SEROLOGICAL NEGATIVE SAMPLES AND PCR RHD STATUS.

Red cell antibodies were found associated with both RHD negative and RHD positive individuals (Table 4.1). Four samples out of the 8 samples that had red cell antibodies in the sera were RHD-positive by PCR typing. However, red cell antibodies were not associated with the indeterminate RHD status (both exon 7 and 10 absent) by PCR.

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Table 4.1: Red cell antibody among RHD serological negative samples and PCR RHD status.

SAMPLE ID	RHD SEROLOGY STATUS	RED CELL ANTIBODY	PCR RHD STATUS
RDN005	Negative	Reactive	Negative
RDN014	Negative	Reactive	Positive
RDN018	Negative	Reactive	Negative
RDN024	Negative	Reactive	Negative
RDN035	Negative	Reactive	Positive
RDN038	Negative	Reactive	Negative
RDN045	Negative	Reactive	Positive
RDN056	Negative	Reactive	Positive

4.6 MULTIPLEX PCR ANALYSIS

From all DNA samples, the 434-bp internal control fragment from the human growth hormone (HGH) gene was amplified (Fig. 4.3). However, none of the six RHD specific exons were amplified from the 34 D+ and 67 D- individuals.

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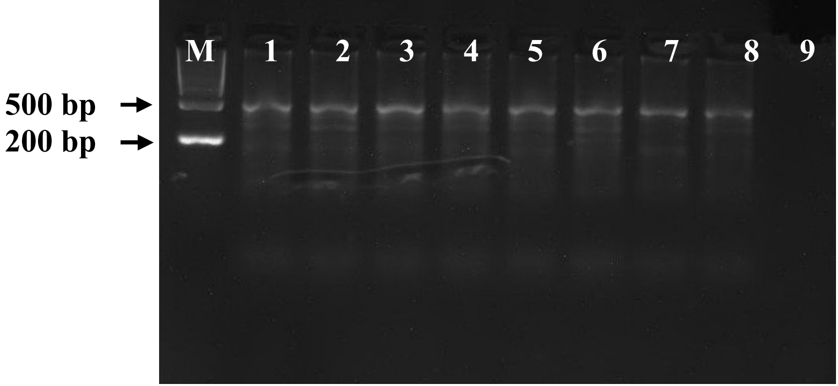

Fig. 4.3: Ethidium bromide stained with 4% agarose gel electrophoregram of multiplex PCR analysis.

Lane M = 100 bp ladder; Lanes 1-8 = samples; Lane 9 = negative control

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CHAPTER FIVE

DISCUSSION AND CONCLUSIONS

5.1 DISCUSSION

This study was to ascertain the molecular RHD status of serologically RHD negative pregnant women in the Greater Accra Region of Ghana. The study also sought to establish the association of antibodies found with the RHD gene status of the participants. Definitive designation of RHD status is crucial in the administration of ant-D immunoglobulin prophylaxis and transfusion cross matching among pregnant women. In the case of RHD variants, distinction between RHD positive and RHD negative phenotype presents a challenge. Some available anti-D reagents can identify these variants whilst others cannot (Wang, Lane and Quillen, 2010; Lukacevic Krstic *et al.*, 2018).

RHD negative phenotype in Europeans is as a result of deletion of the RHD gene (Daniels, 2013; Orzińska *et al.*, 2013). In some individuals especially of the African descent , the RHD gene protein or its fragments are either not expressed or translated on the membrane of the red blood cell (Orzińska *et al.*, 2013). In 82% black Africans, RHD negative phenotype is not as a result of homozygous deletion of the RHD gene, but possession of an RHD variant, RHD pseudogene or RH-CE-D hybrid (Gunel *et al.*, 2011; Szulman *et al.*, 2012).

Molecular screening of the RHD gene reveals large deletions of the D antigen genes (Srivastava *et al.*, 2018). This work showed about 65% deletions of the RHD gene in RHD negative pregnant women. Molecular determination of blood group antigens is straightforward in blood groups that have limited number of alleles. Nevertheless,

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molecular designation of certain blood groups remains a challenge due to variant number of alleles and complexity of interpreting (Fasano, 2016; Fasano and Chou, 2016). Molecular determination of the RH antigens is complicated in certain areas due to high prevalence of the RHD variants (Wang, Lane and Quillen, 2010a; Lukacevic Krstic *et al.*, 2018; Dezan *et al.*, 2017). A large deletion of the RHD gene is one of the main mechanisms for decrease RHD protein expression or total loss of it. Therefore accurate molecular depiction of such deletion is a necessity in RHD genotyping (Li *et al.*, 2008; Sippert *et al.*, 2015; Srivastava *et al.*, 2018).

The RHD gene was detectable in 30% of the study population. In some individuals who are serologically RHD negative, the RHD gene was detected (Okuda *et al.*, 1997). In other studies, individuals were positive for at least one of the three RHD exons (Gowland *et al.*, 2014). With several evidence supporting the fact that, the RHD gene is detectable in serological RHD negative individuals, this work has established that the RHD gene can be detected in RHD negative pregnant women in Ghana.

In 8% of the samples in this study, both exon 7 and 10 of the RHD gene did not amplify. In black Africans, a 37 base pair duplication occurs in intron 4 of the RHD gene giving rise to the RHD pseudogene (RHDΨ) (Reid, 2001). RHDΨ do not have transcript of the RHD protein; the RHD Ψ is serologically typed as RHD negative (Gowland *et al.*, 2014). It estimated to be about 24% in RHD negative blacks (Gowland *et al.*, 2014). The lack of expression of both exon 7 and 10 of the RHD gene can be attributed to presence of the RHDΨ. This study was on a black population, which has a high prevalence of RHDΨ, thus it is very likely that the eight percent (8 %, both exon 7

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and 10 not amplified) found in this study could be the RHDΨ to be confirmed in a bigger study.

In developed countries, anti-D immunoglobulin prophylaxis has reduced alloimmunisation rate to around six per 1000 live births (Kacker *et al.*, 2015). In this study, the risk alloimmunisation was 7.2% (1.625 ± 0.5). In other studies the risk of alloimmunisation was 0.73% (Pal and Williams, 2015). This can be attributed to advanced methods of screening and available data of screening (Mcbain, Crowther and Middleton, 2015). However, in a developing country like Ghana, where data on alloimmunisation events unavailable and lack of robust screening regimes (Osaro and Charles, 2010) its extremely difficult to estimate immunization risk in the population. Besides there is high rate of alloimmunisation in RHD negative individuals than the control population reported elsewhere (Eccles, Crispin and Vanniasinkam, 2015). Similarly, limited access to immunoglobulin prophylaxis could account for the high rate of alloimmunisation generally (Osaro and Charles, 2010). The high prevalence rate of antibody found in this study could be due to a significant proportion of females been alloimmunised(Eccles, Crispin and Vanniasinkam, 2015). Prevalence of red cell antibodies was 1.8% in Asian population and anti-D in RHD negative individuals was 2.9% (Karim, Moiz and Kamran, 2015). The high number of antibodies found in this study is anticipated because, females have high risk of alloimmunisation, particularly RHD negative individuals, with the production of anti-D antibodies (Dhawan *et al.*, 2014; Eccles, Crispin and Vanniasinkam, 2015; Karim, Moiz and Kamran, 2015; Gehrie *et al.*, 2017). Variations in alloimmunisation could be influenced by geographical divergence in blood group antigen expression, especially the Rh blood group (Karim, Moiz and Kamran, 2015).

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Red cell alloimmunisation has been reported to be about 5.6% among thalassemia major patients (Dhawan *et al.*, 2014). In sickle cell patients, red cell alloimmunisation was up to 30% (Gehrie *et al.*, 2017) and the most frequent reported antibody was specific to the Rh blood group. The chances of alloimmunisation to RHD gene is about 1% during first pregnancy or after first pregnancy (Mcbain, Crowther and Middleton, 2015). Alloimmunisation among RHD negative individuals was 10% in Oman (Al-Dughaishi *et al.*, 2016), a rate higher than that of this study. Altuntas proposed that the likely causes of sensitisation includes blood transfusion, feto-maternal haemorrhage relating to child birth, trauma, miscarriages (spontaneous and induced), ectopic pregnancy and other invasive procedures (Altuntas *et al.*, 2012). Based on these findings, the risk of alloimmunisation risk of 7.3% is a expected because of lack of reliable data on alloimmunisations events on this study population. Besides, these findings are comparable to similar studies in other settings.

The limitations of the study were:

1. The study was limited to pregnant women, who are at high risk of alloimmunisation and therefore the rate of alloimmunisation may be higher than anticipated in the general population.
2. The primers used amplified two (2) exons (exon 7 and 10) that are similar to both RHD and RHCE genes.
3. The risk factors for alloimmunisation was not ascertained in this study.
4. The work could not specifically identify the antibodies with the corresponding red cell antigen.

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5.2 CONCLUSIONS

This study has identified 7.3% of pregnant women with unexpected red cell antibodies that are at risk of alloimmunisation. Thirty percent (30%) of the serological RHD negative samples were confirmed as RHD positive by a molecular assay. None of the six RHD specific exons were amplified from the 34 D+ and 67 D- individuals. This work also postulates that there is a possible persistence of the RHD pseudogene in our population. This study has shown that, not all RHD negative pregnant require anti-D immunoglobulin prophylaxis.

It is recommended that:

1. The high prevalence of the RHDΨ in the African population (Gunel *et al.*, 2011; Szulman *et al.*, 2012; Ziza *et al.*, 2017), necessitates the incorporation of primers specific for RHDΨ, when screening for RHD genotypes in an African setting.
2. There is the need for a population based study to genotype and sequence all samples expressing RHD negative phenotype to ascertain the specific RHD variants in the Ghanaian population.
3. All serological reagents should be procured in cognisance with the RHD variants present in the Ghanaian population. The high prevalence of RHD variants in the African population (Denomme *et al.*, 2005; Wang, Lane and Quillen, 2010; Abdelkefi *et al.*, 2014) demands a RHD genotyping and sequencing in pregnant women and blood donors.

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APPENDICES

Appendix I: Antibody screening of RHD negative pregnant women using serum

S/NO	SAMPLE ID	RESULT
1.	RDN001	NON-REACTIVE
2.	RDN002	NON-REACTIVE
3.	RDN003	NON-REACTIVE
4.	RDN004	NON-REACTIVE
5.	RDN005	**POSITIVE**
6.	RDN006	NON-REACTIVE
7.	RDN007	NON-REACTIVE
8.	RDN008	NON-REACTIVE
9.	RDN009	NON-REACTIVE
10.	RDN010	NON-REACTIVE
11.	RDN011	NON-REACTIVE
12.	RDN012	NON-REACTIVE
13.	RDN013	NON-REACTIVE
14.	RDN014	**POSITIVE**
15.	RDN015	NON-REACTIVE
16.	RDN016	NON-REACTIVE
17.	RDN017	NON-REACTIVE
18.	RDN018	**POSITIVE**
19.	RDN019	NON-REACTIVE
20.	RDN020	NON-REACTIVE
21.	RDN021	NON-REACTIVE
22.	RDN022	NON-REACTIVE
23.	RDN023	NON-REACTIVE
24.	RDN024	**POSITIVE**
25.	RDN025	NON-REACTIVE
26.	RDN026	NON-REACTIVE
27.	RDN027	NON-REACTIVE
28.	RDN028	NON-REACTIVE
29.	RDN029	NON-REACTIVE
30.	RDN030	NON-REACTIVE
31.	RDN031	NON-REACTIVE
32.	RDN032	NON-REACTIVE
33.	RDN033	NON-REACTIVE
34.	RDN034	NON-REACTIVE
35.	RDN035	**POSITIVE**
36.	RDN036	NON-REACTIVE
37.	RDN037	NON-REACTIVE
38.	RDN038	**POSITIVE**
39.	RDN039	NON-REACTIVE
40.	RDN040	NON-REACTIVE
41.	RDN041	NON-REACTIVE
42.	RDN042	NON-REACTIVE
43.	RDN043	NON-REACTIVE

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44.	**RDN044**	NON-REACTIVE
45.	**RDN045**	**POSTIVE**
46.	**RDN046**	NON-REACTIVE
47.	**RDN047**	NON-REACTIVE
48.	**RDN048**	NON-REACTIVE
49.	**RDN049**	NON-REACTIVE
50.	**RDN050**	NON-REACTIVE
51.	**RDN051**	NON-REACTIVE
52.	**RDN052**	NON-REACTIVE
53.	**RDN053**	NON-REACTIVE
54.	**RDN054**	NON-REACTIVE
55.	**RDN055**	NON-REACTIVE
56.	**RDN056**	**POSITIVE**
57.	**RDN057**	NON-REACTIVE
58.	**RDN058**	NON-REACTIVE
59.	**RDN059**	NON-REACTIVE
60.	**RDN060**	NON-REACTIVE
61.	**RDN061**	NON-REACTIVE
62.	**RDN062**	NON-REACTIVE
63.	**RDN063**	NON-REACTIVE
64.	**RDN064**	NON-REACTIVE
65.	**RDN065**	NON-REACTIVE
66.	**RDN066**	NON-REACTIVE
67.	**RDN067**	NON-REACTIVE
68.	**RDN068**	NON-REACTIVE
69.	**RDN069**	NON-REACTIVE
70.	**RDN070**	NON-REACTIVE
71.	**RDN071**	NON-REACTIVE
72.	**RDN072**	NON-REACTIVE
73.	**RDN076**	NON-REACTIVE
74.	**RDN078**	NON-REACTIVE
75.	**RDN079**	NON-REACTIVE
76.	**RDN082**	NON-REACTIVE
77.	**RDN083**	NON-REACTIVE
78.	**RDN084**	NON-REACTIVE
79.	**RDN085**	NON-REACTIVE
80.	**RDN086**	NON-REACTIVE
81.	**RDN087**	NON-REACTIVE
82.	**RDN088**	NON-REACTIVE
83.	**RDN089**	NON-REACTIVE
84.	**RDN090**	NON-REACTIVE
85.	**RDN091**	NON-REACTIVE
86.	**RDN092**	NON-REACTIVE
87.	**RDN093**	NON-REACTIVE
88.	**RDN094**	NON-REACTIVE
89.	**RDN095**	NON-REACTIVE
90.	**RDN096**	NON-REACTIVE
91.	**RDN097**	NON-REACTIVE

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92.	**RDN098**	NON-REACTIVE
93.	**RDN099**	NON-REACTIVE
94.	**RDN100**	NON-REACTIVE
95.	**RDN101**	NON-REACTIVE
96.	**RDN102**	NON-REACTIVE
97.	**RDN103**	NON-REACTIVE
98.	**RDN104**	NON-REACTIVE
99.	**RDN105**	NON-REACTIVE
100.	**RDN106**	NON-REACTIVE
101.	**RDN107**	NON-REACTIVE
102.	**RDN108**	NON-REACTIVE
103.	**RDN109**	NON-REACTIVE
104.	**RDN110**	NON-REACTIVE
105.	**RDN113**	NON-REACTIVE
106.	**RDN114**	NON-REACTIVE
107.	**RDN116**	NON-REACTIVE
108.	**RDN117**	NON-REACTIVE
109.	**RDN118**	NON-REACTIVE
110.	**RDN119**	NON-REACTIVE

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Appendix II: Molecular Screening for RHD status

S/NO	SAMPLE ID	RHD EXON 7	RHD EXON 10	NO EXON 7 AND 10	MOLECULAR RHD STATUS
1.	RDN001	+			RHD NEGATIVE
2.	RDN002	+			RHD NEGATIVE
3.	RDN003	+			RHD NEGATIVE
4.	RDN004	+			RHD NEGATIVE
5.	RDN005	+			RHD NEGATIVE
6.	RDN006	+			RHD NEGATIVE
7.	RDN007	+			RHD NEGATIVE
8.	RDN008			+	INDETERMINATE
9.	RDN009	+	+		RHD POSITIVE
10.	RDN010	+			RHD NEGATIVE
11.	RDN011	+			RHD NEGATIVE
12.	RDN012	+			RHD NEGATIVE
13.	RDN013	+	+		RHD POSTIVE
14.	RDN014	+	+		RHD POSITIVE
15.	RDN015	+	+		RHD POSITIVE
16.	RDN016	+			RHD NEGATIVE
17.	RDN017	+			RHD NEGATIVE
18.	RDN018	+			RHD NEGATIVE
19.	RDN019	+			RHD NEGATIVE
20.	RDN020	+			RHD NEGATIVE
21.	RDN021	+			RHD NEGATIVE
22.	RDN022	+			RHD NEGATIVE
23.	RDN023	+			RHD NEGATIVE
24.	RDN024	+			RHD NEGATIVE

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25.	**RDN025**	+			**RHD NEGATIVE**
26.	**RDN026**	+	+		**RHD POSITIVE**
27.	**RDN027**	+			**RHD NEGATIVE**
28.	**RDN028**	+			**RHD NEGATIVE**
29.	**RDN029**	+			**RHD NEGATIVE**
30.	**RDN030**	+			**RHD NEGATIVE**
31.	**RDN031**	+			**RHD NEGATIVE**
32.	**RDN032**	+			**RHD NEGATIVE**
33.	**RDN033**	+			**RHD NEGATIVE**
34.	**RDN034**	+			**RHD NEGATIVE**
35.	**RDN035**	+	+		**RHD POSITIVE**
36.	**RDN036**	+			**RHD NEGATIVE**
37.	**RDN037**	+	+		**RHD POSITIVE**
38.	**RDN038**	+			**RHD NEGATIVE**
39.	**RDN039**	+			**RHD NEGATIVE**
40.	**RDN040**	+			**RHD NEGATIVE**
41.	**RDN041**	+	+		**RHD POSITIVE**
42.	**RDN042**	+			**RHD NEGATIVE**
43.	**RDN043**	+	+		**RHD POSITIVE**
44.	**RDN044**	+			**RHD NEGATIVE**
45.	**RDN045**	+	+		**RHD POSITIVE**
46.	**RDN046**	+			**RHD NEGATIVE**
47.	**RDN047**	+			**RHD NEGATIVE**
48.	**RDN048**	+	+		**RHD POSITIVE**
49.	**RDN049**	+			**RHD NEGATIVE**
50.	**RDN050**	+			**RHD NEGATIVE**
51.	**RDN051**	+			**RHD NEGATIVE**

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52.	**RDN052**	+			**RHD NEGATIVE**
53.	**RDN053**	+			**RHD NEGATIVE**
54.	**RDN054**	+			**RHD NEGATIVE**
55.	**RDN055**	+	+		**RHD POSITIVE**
56.	**RDN056**	+	+		**RHD POSITIVE**
57.	**RDN057**			+	**INDETERMINATE**
58.	**RDN058**			+	**INDETERMINATE**
59.	**RDN059**			+	**INDETERMINATE**
60.	**RDN060**			+	**INDETERMINATE**
61.	**RDN061**	+			**RHD NEGATIVE**
62.	**RDN062**	+			**RHD NEGATIVE**
63.	**RDN063**	+			**RHD NEGATIVE**
64.	**RDN064**	+			**RHD NEGATIVE**
65.	**RDN065**			+	**INDETERMINATE**
66.	**RDN066**			+	**INDTERMINATE**
67.	**RDN067**	+			**RHD NEGATIVE**
68.	**RDN068**	+	+		**RHD POSITIVE**
69.	**RDN069**	+	+		**RHD POSITIVE**
70.	**RDN070**	+			**RHD NEGATIVE**
71.	**RDN071**	+	+		**RHD POSITIVE**
72.	**RDN072**	+			**RHD NEGATIVE**
73.	**RDN076**	+	+		**RHD POSITIVE**
74.	**RDN078**	+			**RHD NEGATIVE**
75.	**RDN079**	+	+		**RHD POSITIVE**
76.	**RDN082**	+	+		**RHD POSITIVE**
77.	**RDN083**	+	+		**RHD POSITIVE**

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78.	**RDN084**			+	**INDETERMINATE**
79.	**RDN085**			+	**INDETERMINATE**
80.	**RDN086**	+			**RHD NEGATIVE**
81.	**RDN087**	+	+		**RHD POSITIVE**
82.	**RDN088**	+			**RHD NEGATIVE**
83.	**RDN089**	+	+		**RHD POSITIVE**
84.	**RDN090**	+			**RHD NEGATIVE**
85.	**RDN091**	+	+		**RHD POSITIVE**
86.	**RDN092**	+	+		**RHD POSITIVE**
87.	**RDN093**	+			**RHD NEGATIVE**
88.	**RDN094**	+	+		**RHD POSITIVE**
89.	**RDN095**	+	+		**RHD POSITIVE**
90.	**RDN096**	+			**RHD NEGATIVE**
91.	**RDN097**	+	+		**RHD POSITIVE**
92.	**RDN098**	+			**RHD NEGATIVE**
93.	**RDN099**	+			**RHD NEGATIVE**
94.	**RDN100**	+			**RHD NEGATIVE**
95.	**RDN101**	+	+		**RHD POSITIVE**
96.	**RDN102**	+	+		**RHD POSITIVE**
97.	**RDN103**	+	+		**RHD POSITIVE**
98.	**RDN104**	+			**RHD NEGATIVE**
99.	**RDN105**	+			**RHD NEGATIVE**
100.	**RDN106**	+			**RHD NEGATIVE**
101.	**RDN107**	+			**RHD NEGATIVE**
102.	**RDN108**	+			**RHD NEGATIVE**
103.	**RDN109**	+	+		**RHD POSITIVE**
104.	**RDN110**	+			**RHD NEGATIVE**

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105.	**RDN113**	+			**RHD NEGATIVE**
106.	**RDN114**	+			**RHD NEGATIVE**
107.	**RDN116**	+			**RHD NEGATIVE**
108.	**RDN117**	+			**RHD NEGATIVE**
109.	**RDN118**	+	+		**RHD POSITIVE**
110.	**RDN119**	+	+		**RHD POSITIVE**

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Appendix III: ETHICAL CLEARANCE CERTIFICATE

UNIVERSITY OF GHANA
SCHOOL OF BIOMEDICAL AND ALLIED HEALTH SCIENCES

14th March, 2017

Ref. No.:...
Mr. Elvis Baseriyi Batuu,
Dept. of Medical Laboratory Sciences,
SBAHS,
Korle Bu.

Dear Mr. Batuu,

ETHICS CLEARANCE

Ethics Identification Number: SBAHS – MD. /10551119/AA/5A/2016-2017.

Following a meeting of the Ethics and Protocol Review Committee of the School of Biomedical and Allied Health Sciences held on Tuesday 14th. March, 2017. I write on behalf of the Committee to approve your research proposal as follows:

TITLE OF RESEARCH PROPOSAL: RHESUS D VARIANTS (WEAK D AND PARTIAL D0 AMONG PREGNANT WOMEN IN THE GREATER ACCRA REGION OF GHANA

This approval requires that you submit three-monthly review reports of the protocol to the Committee and a final full review to the Committee on completion of the research. The Committee may observe the procedures and records of the research during and after implementation.

Please note that any significant modification of the research must be submitted to the Committee for review and approval before its implementation.

You are required to report all serious adverse events related to this research to the Committee within seven (7) days verbally and fourteen (14) days in writing.

As part of the review process, it is the Committee's duty to review the ethical aspects of any manuscript that may be produced from this research. You will therefore, be required to furnish the Committee with any manuscript for publication.

This reviewed report is valid till 31st. August, 2017

Please always quote the ethical identification number in all future correspondence in relation to this protocol.

Thank you.

Yours sincerely,

Dr. S. D. Amanquah
(Chairman, Ethics and Protocol Review Committee)

Cc: Dean
Head, Dept. of Medical Laboratory Sciences
School Administrator

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