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Review Article

Rh D Genotyping in Pregnancy - Present and Future

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Abstract

Background: The RBC's (Red Blood Cell's) antigen can cause alloimmunisation during pregnancy if the fetus inherited the antigen from the father that is not present in the mother. In most of the cases the RBS's antibody are from IgG class and they cross the placental barrier, binding the Fc receptors as an active one way transport from the mother to the fetus and never cross versa.

Aim: To emphasize that methods of genotyping in pregnancy improve the management of sensibilised pregnancies in high risk of HDFN easier and safer minimising the unnecessary procedure. Prove that with genotyping antenatal use of RhIG can be reduced, same as use of RhD-negative blood for transfusion.

Materials and Methods: All pregnant women were tested for ABO, RhD, Rh phenotype, K and screening of alloantibodies in the first trimester of pregnancy. Pregnant women who have the DVI phenotype are typed as D-negative. Pregnant women, 50 in total, with weak expression of D, with score < 2+, including those with DEL phenotype, were tested with a panel of D monoclonal antibodies, commercial kit, or by molecular testing, for RhD variants.

Results: The results that we have obtained show that 17 samples were RhD-negative and 33 samples showed results for weak D: weak D Type 1 (60,6%), Type 2 (12,2%), Type 3 (24,2%) and only 1 pregnant woman was RhD Type 4. The research also included 30 pregnant women where the RhD fetal status had been detected by non-invasive technique from the mother's plasma, by Real time PCR method, between the 12 and 31 gestation week. Acquired results demonstrated that 12 fetuses were female, 16 were male and 3 fetuses were without specified sex.

Conclusion: In our study only 3% of RhD-negative mothers needed RhIG prophylaxis, and 97% were weak D variants 1 - 3 that we can consider as RhD-positive and they didn't need an RhIG prophylaxis, subsequently they can be transfused with RhD-positive blood.

According to the results, we provide RhIG antenatal prophylaxis in 97% unnecessarily and expose the women on human product, with all risk of it. Also, there is a possibility to save a stock of RhD-negative blood in these women, if the transfusion is necessary.

Keywords: *RBC's (Red Blood Cell's); Rh D Genotyping; Pregnancy*

Introduction

The RBC's (Red Blood Cell's) antigen can cause alloimmunisation during pregnancy if the fetus inherited the antigen from the father that is not present in the mother. In most of the cases the RBS's antibody are from IgG class and they cross the placental barrier, binding the Fc receptors as an active one way transport from the mother to the fetus and never cross versa.

HBFN is directly dependant on the development of RBC's antigens on the surface of fetal RBC. The antigens on the RBC's membrane of the newborn are not fully expressed. The antigens as ABH, Lewis, P, Sd^a which are immunomodulator sugars are not well developed immediately after birth. On the other hand, protein antigens Rh, MNS, Duffy, Kell, Kid are completely present. For example, the D antigen

can be proven as soon as the 38th gestation day, and it is a reason for severe HBFN, due to higher sensitivity and high potential of anti-D antibodies that can destroy D-positive fetus RBC [1-4].

Antenatal anti-D prophylaxis (RhIG) was introduced since 1960, noted that there is a significant decrease in the rate of anti-D immunization. By adding RhIG in high risk situations during pregnancy and delivery, there is an aditional decrease in the rate of anti-D immunization and anti-D perinatal mortality to 1,5 - 2% [6]. Further decrease had been noticed with routine antenatal application of RhIG in order to prevent immunisation of undiscovered FMH during the last trimester of pregnancy [5,6]. But even if postnatal and antenatal prophylaxis is combined, 0.1 - 0.3% of the women at risk still create anti-D antibodies [7,8].

RhD (D or RH1), originally identified in 1939, was the first clinically important blood group to be found following the discovery of ABO 39 years earlier.

RHD and *RHCE*, a pair of homologous genes sharing 93,8% homology over all introns and coding exons, encode the antigens of the Rh system. They are located on chromosome 1p36·11 and are closely linked, nevertheless are in opposite orientation: 5′-*RHD*-3′-3′-*RHCE*-5′ [9]. Each gene consists of 10 exons that encode 417-amino acid polypeptides, although the N-terminal methionine is cleaved from the mature proteins [10]. The RhD and RhCE proteins differ by between 31 and 35 amino acids, depending on the *RHCE* allele. They are hydrophobic molecules making six potential external loops with internal N- and C-termini as a result of spanning the red cell membrane 12 times (Figure 1). Atypically for red cell membrane proteins, they are not glycosylated. Homology modelling based on crystal structures of bacterial homologues of the Rh proteins and on the human non-erythroid Rh family glycoprotein RhCG predict the presence of an extracellular vestibule in the regions of the third and fourth external loops and sixth, seventh, and eighth membrane spanning domains that penetrates the cell membrane permitting access to IgG antibodies [10-12].

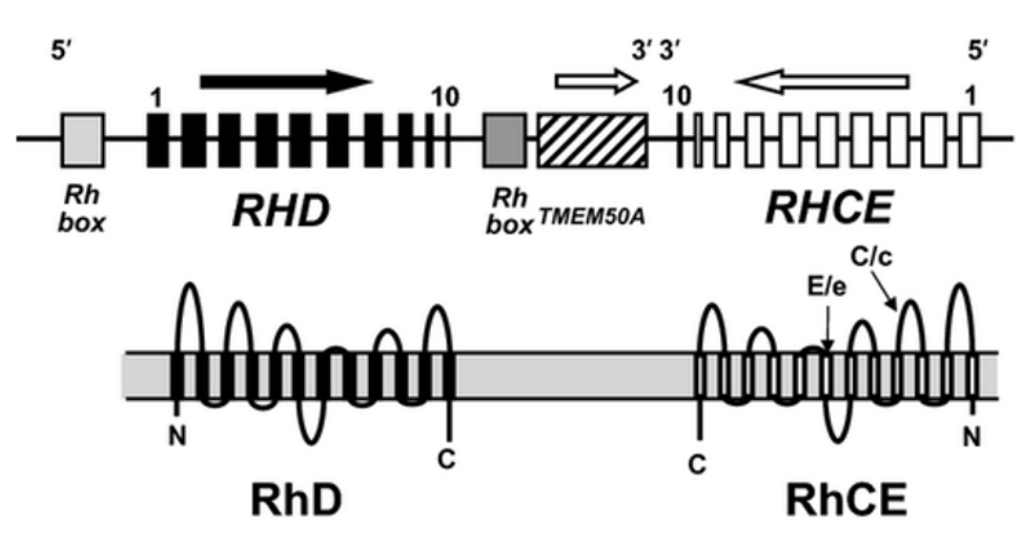


Figure 1: *RhD and RhCE proteins with internal N- and C-termini and six potential external loops.*

The D-phenotype usually results from a complete absence of the RhD protein, explaining the high immunogenicity of D. The RhCE protein is almost always present. In Caucasians, the usual cause of the D- negative phenotype is homozygosity for a complete deletion of *RHD* (Figure 2).

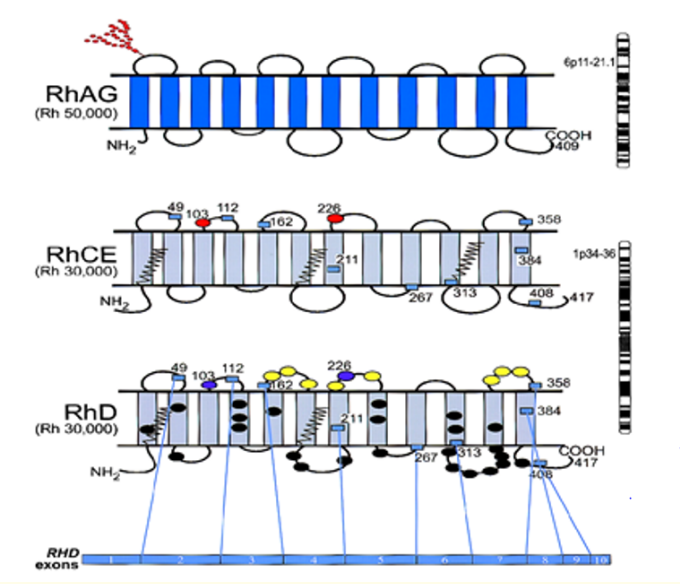


Figure 2: *Scheme display of the RH gene.*

Since the cloning of *RHD* and the elucidation of the molecular bases for the D polymorphism, it has become common practice to predict D phenotype from genomic DNA. The most common molecular background to the D- negative phenotype is homozygosity for a deletion of *RHD*, so the basis of molecular D typing is amplifying part of *RHD* to assess whether the gene is present.

More than 200 different RHD alleles identified to date encode single or multiple amino acid changes in RhD. These changes can cause decrease amount of protein in membrane or can alter protein and abolish or create novel epitopes.

The cause of variability of expression of D antigen can be caused from changes in D epitopes on Rhce protein, different genetic RHD alleles. Additionally, different reagents and methods used in the laboratory or a combination for detection of D antigen can be the reason for variability of D antigen.

Materials and Methods

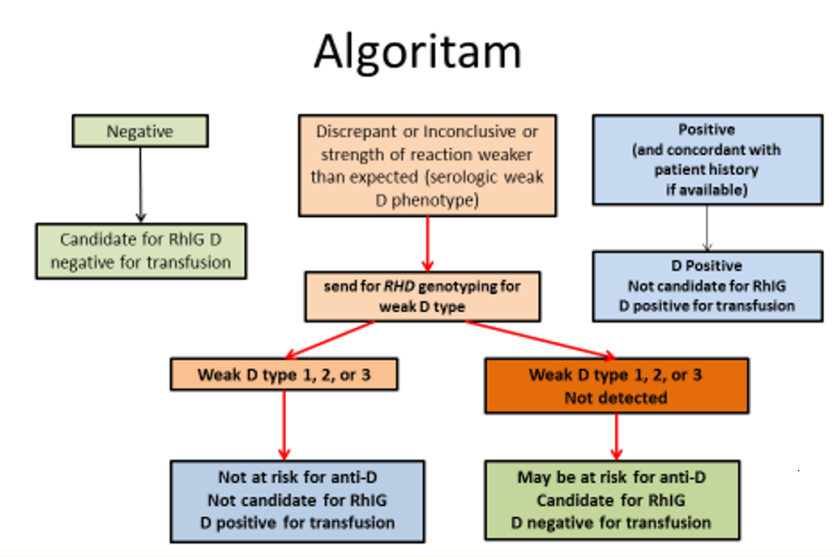
The aim of immunohaematology testing in pregnancy is to determinate the RhD-negative pregnant women who need RhD prophylaxis, to identify presence of significant alloantibodies for management of sensibilised pregnant women and to identify blood group for possible necessity of transfusion. The recommended practice in our institution is to test all pregnant women for ABO, RhD blood groups (Bio-Rad ID-Card: DiaClon A, B, DIV-, Reverse Grouping) and screening of alloantibodies in the first trimester of pregnancy (Bio-Rad ID-Card: Coombs Anti-IgG, Dia-Screen I-II-III-IV-VP-VIP). In all pregnant women with positive screening results, identification of antibodies is performed (Bio-Rad ID-Dia Panel IAT, ID-Dia Panel IAT-P, Ortho Clinical Diagnostics 0,8% Resolve Panel C untreated, 0,8% Resolve Panel C treated). We use the antiglobulin test with potent monoclonal anti-D for all RhD-negative pregnant women, but these reagents are selected to detect all but the weakest examples of D and to give a negative reaction with DVI red cells.

Consequently, pregnant women who have the DVI phenotype and are, therefore, prone to make anti-D, are typed as D-negative and treated as D-negative for blood transfusion and administration of anti-D immunoglobulin during pregnancy and following delivery of a D-positive baby. Pregnant women with very weak expression of D, including those with DEL phenotype, will also be typed as D-negative,

until the cause of the aberrant result is resolved. Further testing with a panel of D monoclonal antibodies (Bio-Rad ID-Partial RhD Typing Set) commercial kit, will assist in the identification of some of the more common variants, especially weak D types 1 and 2. More definitive identification can be achieved by molecular testing, either with kits or arrays designed to identify many or most of the known variants, or by sequencing *RHD*, which will also identify new variants. Once the variant has been identified, then a policy must be in place for transfusion and giving anti-D prophylaxis.

In our study we provide D genotyping in all pregnant women with serology discrepancies or with weak reaction for D antigen, 50 in total. For serology (ABO, Rh phenotype, K, IAT) we used two different types of microagglutination technique: microgel (BioRad) and magnetic pearls (column agglutination technology - CAT) of the ORTHO BioVue® system. For all RhD-negative women we performed antiglobulin test. All weak reactions with score < 2+ were tested for RhD variants. In our study we used commercial blood group genotyping platforms apply polymerase chain reaction with sequence-specific primers and gel electrophoresis which can distinguish an optimal number of variants Innotrain (Kronberg, Germany).

According to the algorithm we provided, all RhD- negative pregnant women with RhD-positive biologic father of the baby, were suggested to provide RhIG antenatal and postnatal prophylaxis if the newborn is positive. They are also candidates for RhD-negative transfusion if necessary. RhD-positive pregnant women were not candidates for RhIG prophylaxis, but candidates for RhD-positive transfusion. Those that manifested discrepancy or inconclusiveness or having weaker reaction similar to a serologic weak D phenotype, were sent for RhD genotyping (Table 1).



Figure

Pregnant women	Weak D Type 1	Weak D Type 2	Weak D Type 3	Weak D Type 4
Number	20	4	8	1
% of total	60,6	12,2	24,2	3,0
Risk of anti-D	No	No	No	Yes
RhIG	Not candidate for RhIG			Candidate for RhIG

Table 1: *RHD genotyping report for weak D.*

The results that we have obtained show that 17 samples were RhD-negative and 33 samples showed results for weak D. Majority of D alleles, with single point mutation were weak D Type 1 (60,6%), Type 2 (12,2%) and Type 3 (24,2%). These pregnant women are not at risk for clinical significant anti-D antibody and they can be transfused with RhD-positive blood units. Only 1 pregnant woman was RhD Type 4 and she was at risk for clinical significant anti-D antibody, she was a candidate for RhIG prophylaxis and of course she can be transfused only with RhD-negative blood units.

The facts indicate that RhD alloimmunisation remains a significant problem in any country besides the implementation of antenatal and postnatal prophylaxis.

Therefore, probably the most important application of predicting D phenotype from genomic DNA, at least in Europe, for determining fetal D type in D- negative pregnant women with anti-D, is to determine whether the fetus is at risk of HDFN. This testing is performed on cell-free fetal DNA in the maternal plasma.

The research that was implemented included 30 pregnant women where the RhD fetal status had been detected by a Real Time PCR technique from the mother's plasma. Usefulness of determination of fetal RhD status is only in case of determination of the RhD zygote type of the biological father, which should be RhD heterozygote.

The samples of pregnant women were tested by Real time PCR method, between the 12 and 31 gestation week. Each plate for real-time PCR amplification included RHD5, RHD7 and CCR5 amplification on pregnant women samples, RHD negative control and controlling genome DNA (Promega) for the generation of standard curves.

Acquired results demonstrated that 12 fetuses were female,16 were male and 3 fetuses were without specified sex. All of the biological fathers, that were included in the study, were heterozygote for the RhD antigen: 15 of them with Rh phenotype DCe/dce, 6 with DcE/dce phenotype, 5 with Dce/dce, 2 with DcE/dcE and 1 with DCe/dCe Rh phenotype. Obtained results for fetal RhD status demonstrate that 9 fetuses were RhD- negative and 21 RhD-positive.

Since we have taken into consideration that negative results might be a consequence of a lack of fetal DNA in the first trimester, we repeated the tests in women that had negative results after 2-3 weeks, no later than 20th gestation week.

Serologic immunohematology analysis were performed at 27 newborn babies, to confirm the fetus RhD status obtained by a PCR technic.

All of the results that were fetal RhD positive, showed positive results with serological tests in newborns. From 7 fetuses with RhD negative results, there were 6 RhD negative babies and one RhD positive baby born.

Discussion

It has been a common practice that weak D patients should receive D-positive red cells, to conserve stocks of D-negative blood, and that partial D patients should be given D-negative red cells. In addition, partial D pregnant women would be given anti-D immunoglobulin prophylaxis, whilst weak D women would not. This policy is based on the premise that weak D patients cannot make anti-D, whereas partial D patients can. This is a flawed logic for reasons already given and such a policy is not recommended.

Pregnant women and patients with weak D types 1, 2, and 3 make alloanti-D only extremely rarely, if at all. To conserve stocks of D-negative red cells, the recommendations [17] and the policy that has now been adopted in England, suggest that individuals with those variants have to be treated as D-positive. Any other D variant can be considered as D-negative, but it will have only a minimal effect on D-negative stocks as those are relatively rare.

The percentage of RhD-negative fetuses moves around 35% and 40%, depending on the literature.

In order to confirm if the fetal DNA is present or the result is false negative, the male DNA can be examined, as that can occur in half of the cases. There is a possibility to confirm fetal DNA, in cases of negative *RHD* and Y chromosome with a specific sequence made of a set of polymorphic markers.

The non-invasive fetal RhD genotyping was initially used only for D allosensitized pregnant women and today is implemented as a routine in several European states.

If RhD sensitised pregnant women have a confirmation of fetal RhD-negative status, there is no risk of HBFN and invasive monitoring will be avoided.

However, if the fetus inherits the RhD antigen, there is a high risk of HBFN with a need of multitude of immunohematology tests and invasive monitoring of the fetus.

Since 2010, Denmark and Holland introduced a national program for fetal RhD screening in order to apply immunoprophylaxis at not immunised D-negative pregnant women [13]. Recently, routine antenatal anti-D prophylaxis became a standard care for D- pregnant women in many developed countries. Antenatal prophylaxis and genotipisation of fetal RhD status combined to postnatal prophylaxis led to further reduction of maternal immunisation for more than 50% [14-16].

Attention should certainly be paid to unnecessary use of antenatal prophylaxis in women who carry RhD-negative fetus.

There is a possibility for non-invasive genotyping analysis in determination of some other clinically relevant red blood cell antigens. It is an advantage that non-invasive fetal RhD genotyping represents a noninvasive method for determination of the fetal RhD status by analysing the mother's plasma in corelation with the invasive method of amniocentesis and cordocentesis which have been used for decades.

Conclusion

In our study only 3% of RhD-negative mothers needed RhIG prophylaxis, and 97% were Weak D variants 1-3 that we can consider as RhD-positive and they didn't need an RhIG prophylaxis, subsequently they can be transfused with RhD-positive blood. In that way we can save RhD-negative blood reserves as well as RhD immunoglobulin.

In pregnant women, known to have D variant red cells other than weak D types 1-3, antenatal and postnatal prophylaxis should be carried out, because passive anti-D from the product will not bind to the mother's own D variant cells and should be available to suppress immunization [18]. This is particularly important in DVI mothers, whose red cells lack most D epitopes, although in most cases DVI mothers will be routinely typed as D-negative and receive prophylactic anti-D automatically.

There is little information about immunization of D-negative women by D variant fetuses. Although there is no evidence that DVI and other weak forms of D can immunize a D-negative mother during pregnancy or at delivery, logic might deem that neonatal (umbilical cord) D typing be performed with reagents that detect DVI, so that the mother would receive anti-D immunoglobulin if the baby has DVI red cells. That is, neonates would be treated like blood donors for D typing. This is the policy in the Netherlands (http://www.diliguide.nl/document/7584) and the USA [19], but in the UK the BCSH guidelines [20] recommend that the same reagents are used for testing the mother and the cord, in order to avoid any confusion leading to the mother's red cells being tested with the wrong reagents.

Our study, for Non-invasive RhD typing of the fetus, showed that 30% of RhD negative pregnant women receive antenatal RhIG prophylaxis, and have no need of it. The other advantage is that these pregnant women can be saved from unnecessary testing, since there is no risk of HBFN. We must certainly think of the fact that there is a worldwide shortage of RhD immunoglobulin.

It can be said that a revolution has been achieved, in the management of prenatal diagnosis in many aspects. Fetal RhD typing has already made management of pregnancies of high risk of HDFN easier and safer, as it can be determined that the need for invasive testing and potentiate pregnancies need close monitoring.

Possibility to have a non-invasive fetal RhD typing as a routine will lead to many advantages as less exposure of pregnant women to human blood products, than reduced administration of anti-D products, and decreased economic costs to health service providers. It must be emphasized that RhD-negative pregnant women can be withheld from the method, roughly up to 40% who have a D-negative fetus, thus avoiding unnecessary exposure to blood products.

Unfortunately, if only serological testing were used, this policy is difficult to apply, because identification of variants is often not possible.

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