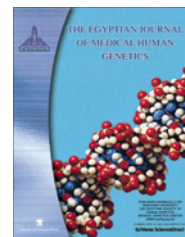




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ORIGINAL ARTICLE

## Correlation between the RhD genotyping and RhD serotyping in isoimmunized pregnancies

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### KEYWORDS

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Rh negative

**Abstract** Alloimmunisation was one of the most important causes of perinatal mortality and morbidity by the middle of the last century. The objective of the present study was to investigate the presence of the RHD gene in fetal cells (amniocytes) obtained from amniotic fluid by genotyping to compare it with the RhD serotyping. Also to correlate the presence of RhD gene with the neonatal outcome. This work was carried out at Maternity hospital and Medical Genetics center, while PCR testing was done at the Medical Research center, Faculty of Medicine, Ain Shams University in the period from 2008 to 2010. The present study included recruiting of 20 RhD negative (sensitized to the RhD antigen) pregnant mothers. The entire study group was subjected to complete general, obstetric and a detailed obstetric ultrasonographic examination. Rh typing and indirect Coomb's test were also done. Amniocentesis was performed with a 20-gauge needle under continuous ultrasound guidance. RhD serotyping of the fetuses showed that, 14 fetuses (70%) were positive and six fetuses (30%) were negative. While using RhD genotyping 13 cases (65%) were positive and seven cases (35%) were negative ( $P$  value = 0.002). Among fetuses positive for RhD genotyping six fetuses (46%) had received postnatal treatment, while among fetuses negative for RhD genotyping, neither of them had received postnatal treatment ( $P$  value = 0.032), which is statistically significant. From the present study we can conclude that, the identification of an

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antigen-negative fetus on the basis of the blood group genotype provides significant advantages in managing the pregnancy at risk for HDFN.

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## 1. Introduction

Alloimmunisation was one of the most important causes of perinatal mortality and morbidity by the middle of the last century. It was affecting 1–2% of all pregnancies and approximately 50% of Rhesus (Rh) immunized pregnancies were lost [1]. This was reduced by 100-fold in the last decades using prophylactic immunoglobulin and fetal therapy procedures, particularly an intrauterine intravascular transfusion (IUIVT) [2]. Today the incidence of hemolytic disease of the newborn secondary to Rh disease is approximately one to six cases per 1000 pregnancies [3]. Despite the development and implementation of Rh(D) immune globulin prophylaxis, maternal Rh alloimmunization is still a cause of erythroblastosis fetalis and hemolytic disease of the newborn. However, with appropriate monitoring and intervention, hemolytic disease of the fetus and newborn can be treated successfully in almost all cases. The outcome of alloimmunized pregnancies is generally very good, with no long-term sequelae in offspring [4].

Maternal alloimmunization, also known as isoimmunization, occurs when a woman's immune system is sensitized to foreign erythrocyte surface antigens, stimulating the production of immunoglobulin G (IgG) antibodies [5]. Antenatal determination of fetal blood group is important in pregnancies with a significant risk of hemolytic anemia due to maternal alloimmunization. Evaluation for the presence of maternal anti-D antibody should be undertaken at the first prenatal visit [6]. First-time sensitized pregnancies are followed with serial maternal titers and, when necessary, serial amniocenteses is indicated to detect fetal bilirubin. The bilirubin level is quantified by spectrophotometry and expressed as the change in optical density at a wavelength of 450 nm ( $\Delta OD_{450}$ ); the  $\Delta OD_{450}$  values are then plotted on a chart devised by Liley to estimate the severity of anemia [7]. To reduce the risk of Rh alloimmunization in a subsequent pregnancy, RhD-negative women are given anti-RhD antibodies after miscarriage, the birth of an RhD-positive baby, or any obstetrical procedure that may cause fetomaternal hemorrhage [8].

Among the more than 50 different antigens capable of causing maternal alloimmunization and fetal hemolytic disease, the Rhesus (Rh) blood group system is the most common. The Rh blood-group antigens are carried by a series of at least three homologous but distinct membrane-associated proteins [9]. Two of these proteins have immunologically distinguishable isoforms designated C, c and E, e. Of note, The principal protein, D, has no immunologically detectable isoform d. Therefore, the presence or the absence of the RhD gene in the genome determines the genetic basis of the polymorphisms associated with Rh positivity and Rh negativity.

The D antigen of the Rh blood group system (RhD) causes most cases of severe hemolytic disease [10]. The incidence of fetuses at risk for anemia due to maternal alloimmunization to red cell antigens has decreased dramatically since the institution of routine anti-D immune globulin (RhoGAM) prophylaxis for Rh-negative women in the 1960s. A review of birth certificate

data in 2003 reported the incidence of Rh sensitization to be approximately 6.8 per 1000 live births [11].

The prevalence of the RhD-negative blood type is dependent on ethnicity with whites having the highest prevalence, while Asians and American Indians having the lowest. Of the existing Rh antigen, the D antigen is the most immunogenic. Approximately 10% of pregnancies in white women are Rh incompatible [12]. However, because the risk of alloimmunization in a susceptible RhD-negative woman is significantly affected by several factors, less than 20% of RhD-incompatible pregnancies actually lead to maternal alloimmunization. These factors include the volume of fetomaternal hemorrhage, the degree of maternal immune response, concurrent ABO incompatibility, and fetal homozygosity versus heterozygosity for the D antigen [13].

Two genes have been identified on the short arm of chromosome 1 that encode the major Rh antigen groups (c/C, D, e/E): RHD and RHCE, with differential protein production in the latter is likely to be due to alternative splicing. Inheritance of these so-called Rh locus genes is closely linked, thus allowing estimations of paternal heterozygosity at the Rh D locus to be made using gene frequency tables that incorporate data on paternal ethnicity, blood type, and number of previous RhD-positive infants [14].

Causes of maternal alloimmunization include: Blood transfusion, fetomaternal hemorrhage (antepartum, intrapartum), abortion (therapeutic, spontaneous), ectopic pregnancy, placental abruption, abdominal trauma, obstetric procedures (amniocentesis, chorionic villus sampling (CVS), percutaneous umbilical blood sampling, manual removal of the placenta) [15].

IgG antibody-mediated hemolysis of fetal erythrocytes, known as hemolytic disease of the fetus and newborn (HDFN), varies in severity and can have a variety of manifestations [16]. The degree of anemia can range from mild to severe with associated hyperbilirubinemia and jaundice. In severe cases, hemolysis may lead to extramedullary hematopoiesis and reticuloendothelial clearance of fetal erythrocytes. This may result in hepatosplenomegaly; decreased liver function; and ensuing hypoproteinemia, ascites, and anasarca. When accompanied by high-output cardiac failure and pericardial effusion, this condition is known as hydrops fetalis. Without intervention, this syndrome is often fatal. Intensive neonatal care, including emergent exchange transfusion [17].

Assessment of fetal RhD status is critical in determining whether a pregnancy in an alloimmunized woman is at risk for the development of hemolytic disease of the fetus and newborn. Once a fetus is found to be at risk (i.e., RhD positive), the goals of managing the alloimmunized pregnancy are twofold. First is the detection of fetal anemia prior to the occurrence of fetal compromise. After detection, the goal is to minimize fetal morbidity and mortality by correcting this anemia until fetal lung maturity and delivery can be achieved. Because of the potential need for invasive diagnostic and therapeutic procedures, pregnancies complicated by erythrocyte

alloimmunization should be managed by maternal-fetal medicine specialists [18].

In fact, Rhesus antibodies when managed by close monitoring, routine antenatal anti-D prophylaxis (RAADP) and timely delivery can lead to favorable outcome among RhD-negative women, also it can prevent sensitisation and hence prevent hemolytic disease of the newborn (HDN) [19].

The objective of the present study was to investigate the presence of the RHD gene in fetal cells (amniocytes) obtained from amniotic fluid by genotyping to compare it with the RhD serotyping. Also to correlate the presence of RhD gene with the neonatal outcome.

## 2. Subjects and methods

This work was carried out at Maternity hospital and Medical Genetics center, while PCR testing was done at the Medical Research center, Faculty of Medicine, Ain Shams University in the period from 2008 to 2010. The present study included recruiting of 20 RhD negative pregnant mothers.

### 2.1. Subjects

Mothers eligible to be included were: RhD negative mothers (mothers sensitized to the RhD antigen and multipara pregnant women). The mean gestational age at which amniocentesis was done is  $26 \pm 1.87$  weeks, with minimum gestational age 19 weeks and maximum gestational age 32 weeks. While, non-sensitized mothers, mothers having nonimmune hydrops foetalis and primigravida pregnant women were excluded.

An informed consent was obtained from all the cases.

### 2.2. Methods

The entire study group was subjected to the following:

- (1) Full detailed medical history and history suggestive of haemolytic disease in previous pregnancies, including miscarriages, ectopic pregnancy, stillbirths, previous hydrops, previous intrauterine blood transfusion, early neonatal deaths, neonatal exchange transfusion and history of maternal blood transfusion.
- (2) Complete general, obstetric and a detailed obstetric ultrasonographic examination.
- (3) Blood and biochemical tests including blood grouping, Rh typing and indirect Coomb's test.

#### 2.2.1. Sampling procedure

Amniocentesis was performed with a 20-gauge needle under continuous ultrasound guidance. Since the majority of partial D phenotype involves hybrid Rh genes; where exchange of DNA has occurred between the RHD and RHCE genes. RhD genotyping, if directed to these regions of exchange, would incorrectly type some partial D phenotypes as D negative as stated by Santanu and Samia [19], therefore we studied a specific sequence to RHD gene (exon 10). Amplification of DNA by polymerase chain reaction (PCR) of a unique sequence of the RHD gene was performed on AF after DNA extraction. Results were compared to the neonatal RhD serologic typing.

The samples were transferred to the Medical Research center (MRC) within half an hour.

In 16 cases the procedure was also performed for the management of suspected alloimmunization in RhD negative mothers and in four cases for fetal karyotyping.

#### 2.2.2. DNA preparation

Amniotic fluid was centrifuged at  $10,000 \times g$  for 10 min. The resulting sediment cells were suspended in phosphate-buffered saline solution. The centrifugation and washing procedures were repeated twice. After the third centrifugation, the cell pellet was incubated with 400 microl of buffer containing 20 mmol/L TRIS (pH 8), 50 mmol/L potassium chloride, 1.5 mmol/L magnesium chloride, 0.5% polysorbate 20 (Tween 20) and 250 mg of proteinase K per milliliter. After incubation at 37 °C for 3 h or at 55 °C for 1 h, the protease was heat inactivated and a 2-microl aliquot was used for PCR amplification.

#### 2.2.3. Polymerase chain reaction

The RhD gene specific DNA fragments were amplified using PCR assay.

The following primers were used:

A3 (5'TAAGCAAAAGCATCCAA3') sense primer, position 1252–1268).

A4 (5'ATGGTGAGATTCTCT3') antisense primer, position 1437–1422).

The nucleotide sequences of the primers used were deduced from RhIX cDNA [20] and from RhXIII cDNA (Le Van Kim) [21].

The primer pairs amplify a 186 base-pair (bp) fragment at exon 10, specific for the RhD gene. PCR was performed in 20-microl reaction mixtures. Thirty cycles of denaturation for 1 min at 92 °C, primer annealing for 1 min at 49 °C, and primer extension for 1 min at 72 °C were performed, followed by a final primer extension for 9 min at 72 °C. The reaction products were separated by electrophoresis with a 3% agarose gel containing 0.5 µg of ethidium bromide per milliliter and were visualized under ultraviolet light.

Photographs were taken using polaroid camera. The detection of PCR products (186 bp) indicated an RhD positive fetus, while the lack of the RhD-specific fragment indicated an RhD negative status.

The turn around time of the PCR was one to two days.

#### 2.2.4. Serologic RhD typing

The results of PCR analysis were compared with those of RhD serotyping, which was done on fetal blood obtained by fetal blood sampling procedures or on cord blood samples at birth.

## 3. Results

The results of the present study could be summarized in the following points:

- (1) RhD serotyping of the fetuses showed that, 14 fetuses (70%) were positive and six fetuses (30%) were negative. While using RhD genotyping 13 cases (65%) were positive and seven cases (35%) were negative

- ( $P$  value = 0.002). This data revealed high significant correlation between RhD genotyping and RhD serotyping.
- (2) Among RhD negative fetuses, the mean value of hemoglobin was  $14.8 \pm 3.9$  g%, while among RhD positive fetuses it was  $10.6 \pm 4.7$  g%. There was a statistical difference between the two values ( $P$  value = 0.050).
  - (3) Among fetuses positive for genotyping; 11 fetuses (85%) were positive for Coomb's test and two fetuses (15%) were negative, while among fetuses negative for genotyping; six fetuses (86%) were negative for Coomb's test and one fetus (14%) was positive ( $P$  value = 0.002) indicating high significant correlation between RhD genotyping and direct Coomb's test.
  - (4) Among fetuses positive for RhD genotyping six fetuses (46%) had received postnatal treatment, while among fetuses negative for RhD genotyping, neither of them had received postnatal treatment. ( $P$  value = 0.032) which is statistically significant.
  - (5) Among fetuses positive for RhD genotyping, seven fetuses (54%) were admitted to neonatal intensive care unit (NICU), four fetuses (31%) were born dead (IUFD) and two fetuses (15%) were normal. While among fetuses negative for RhD genotyping six fetuses (86%) were normal; only one fetus (14%) was born dead. ( $P$  value = 0.007) which is highly significant.
  - (6) Among fetuses positive for RhD genotyping, five fetuses (38%) had hydropic changes, while among fetuses negative for RhD genotyping, only one fetus (14%) had hydropic changes. ( $P$ -value = 0.260) which shows no significant correlation.
  - (7) Our results revealed, no statistical significance between level of anti-D titer and the RhD genotyping ( $P$  value = 0.159).

#### 4. Discussion

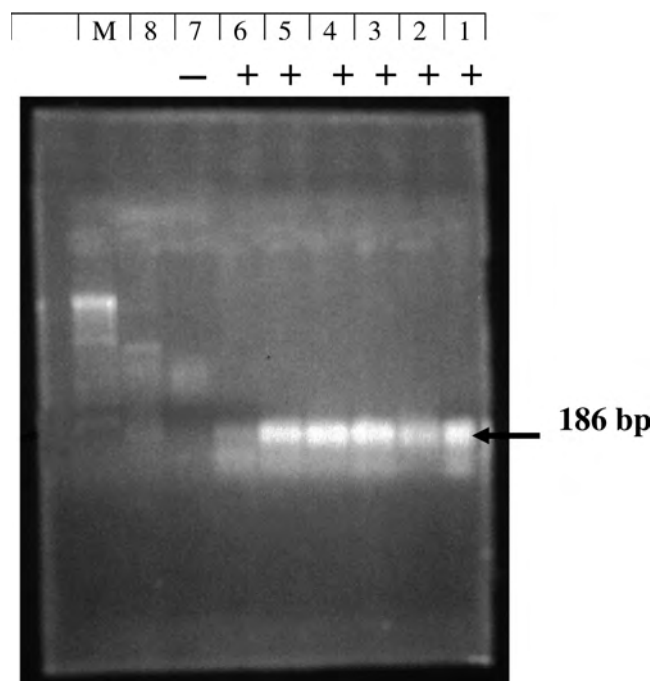
Red cell alloimmunization is the production of antibodies targeted against red blood cells. This occurs in an individual when they are exposed to foreign red cell antigens. There are over 400 red cell antigens that have been identified [22]. If a sensitized pregnant woman has red cell antibodies directed against a specific antigen present on the fetal red cells, several obstetrical complications may occur from transmission of these antibodies across the placenta to the fetus. This may result in fetal anemia, hyperbilirubinemia, hydrops fetalis and possibly fetal death [23]. An RhD-negative mother is at risk of RhD alloimmunization (sensitization) if the fetus is RhD-positive. Without immunoprophylaxis, this would occur in 12–16% of pregnancies in RhD-negative women. The risk of alloimmunization of an RhD-negative mother with an RhD-positive infant is 16% overall, 2% would occur antepartum, 7% would occur by 6 months postpartum and 7% would be 'sensitized'. Women who are sensitized have anti-D antibodies produced at undetectable levels during or after the index pregnancy, but antibodies are identified early in a subsequent pregnancy [24].

In the present study our results showed that among 20 fetuses who have been included, 13 fetuses were correctly typed as RhD positive, six fetuses were typed as RhD negative and one was incorrectly typed as RhD negative; in this case the fetus had severe hydrops fetalis and died in utero at 28 weeks.

In comparison to our results, Lighten et al. [25] published a study on 135 RhD-negative women, who were undergoing amniocentesis for management of suspected alloimmunization. Genotyping was done on specific portions of the Rh D and Rh CcEe genes by polymerase chain reaction; its results were compared with the serological fetal RhD type. Thirty-six were typed RhD negative by both techniques, and 98 fetuses were serologically typed as RhD positive; of these, 96 were correctly typed as RhD positive and two were incorrectly typed as RhD negative, in one of these cases the fetus had mild Rh alloimmune disease and required exchange transfusion at birth. In the second case the fetus had severe hydrops fetalis and died in utero at 28 weeks. Deoxyribonucleic acid isolated from fetal blood was tested with the same polymerase chain reaction technique after delivery, and in both cases the fetus was correctly typed as RhD positive.

It has been concluded from that study that prenatal fetal RhD typing by polymerase chain reaction with amniotic fluid cells is accurate and reliable. In sensitized pregnancies it allows early management of Rh disease and avoids invasive procedures in RhD-negative fetuses. In non-sensitized pregnancies it avoids the use of anti-RhD immunoglobulin after invasive procedures or during pregnancy.

Goebel et al. [26] retrospectively examined the diagnostic accuracy of prenatal RhD blood type genotyping on amniotic fluid, using a combination of two polymerase chain reaction (PCR) methods in daily practice and concluded that prenatal diagnosis of the fetal RhD blood type with PCR from amniotic fluid is highly accurate and associated with a minimal sensitivity of 99.5% and a minimal specificity of 98.6%. Although no



**Figure 1** Agarose gel electrophoresis analysis of PCR products represents the RhD gene amplified from amniotic fluid sample using marker 1× (M). Samples lanes from 1 to 6: indicate positive samples of RhD gene. Sample lane no. 7: indicate RhD negative gene.



discordance between both PCR methods occurred, there were nine out of 927 cases differing results between PCR and serologic status were encountered (see Fig. 1).

Chan [27] had evaluated the effectiveness of a testing strategy of the PCR whereby PCR was not only performed to determine the presence/absence of the RHD gene, but also used to assess the D gene copy number (zero, one or two RhD genes) in family studies for at risk pregnancies. Two discrepant results occurred in one family: the father carried one normal D gene and one D gene variant where PCR was tested to be positive using exon 4 but negative using exon 7. One of a pair of dizygotic twins inherited this abnormal D gene and was mildly affected by HDN. The concordance rate between RhD genotypes from amniotic fluid and RhD serotypes from cord blood was 100%, While in our study the sensitivity of detecting the presence or absence of RHD gene using PCR amplification of exon 10 region only was ~92% the concordance was 95%.

However in another study Van Den Vayver and Moise [28] studied 500 cases in which four different sets of oligonucleotide primers were used. The sensitivity and specificity of PCR typing were 98.7% and 100%, respectively, and the positive and negative predictive values were 100% and 96.9%, respectively (see Tables 1 and 2).

In the present study using PCR to amplify a unique sequence of the RhD gene at exon 10 only showed concordance of 95%. While in a retrospective study Pereira et al. [29] evaluated the efficacy and accuracy of the multiplex polymerase chain reaction (PCR) amplification, for early detection of

**Table 2** Shows sensitivity, specificity, positive predictive value, negative predictive value and accuracy of RhD genotyping using PCR analysis.

Variables	Percentage
True positive	13
True negative	6
False positive	0
False negative	1
Sensitivity	92.9
Specificity	100
PPV	100
NPV	85.7
Accuracy	95

fetuses at risk for hemolytic disease in the population living in Portugal included 2030 uncultured amniotic fluid samples and 2012 blood samples from the respective RhD-negative pregnant women were studied by multiplex PCR of intron 3/ intron 4, exon 7 and 3'UTR (the 3-untranslated region). Serologic RhD blood groups were determined in the cord blood, after birth, for quality control. The results showed 99.5% concordance between the molecular and serologic RhD typing. While among the 2012 serologic RhD-negative mothers, 26 had an RhD-positive allele (1.3%).

In the present study, it was found that RHD genotyping by PCR is a valuable method of antenatal detection of RhD typing in Rh alloimmunised pregnancies with sensitiv-

**Table 1** Characteristic findings of the studied fetuses.

RhD genotype of the fetuses							
Variables	-ve		+ ve		Total		P-value
	No.	%	No.	%	No.	%	
	7	35	13	65	20	100	
Mean HB%	14.8 ± 3.9		10.6 ± 4.7				0.050*
Coomb's test							
-ve	6	86	2	15	8	40	0.002**
+ ve	1	14	11	85	12	60	
Postnatal treatment (phototherapy and exchange transfusion)							
-ve	7	100	7	54	14	70	0.032*
+ ve	0	0	6	46	6	30	
Neonatal condition							
Normal	6	86	2	15	8	40	0.007**
NICU	0	0	7	54	7	35	
Died	1	14	4	31	5	25	
Ultrasound finding (hydrops fetalis)							
-ve	6	86	8	62	14	70	1.266
+ ve	1	14	5	38	6	30	
RhD serotype (fetal and neonatal samples)							
-ve	6	30	0	0	6	30	0.002**
+ ve	1	5	13	65	14	70	
Anti-D titer							
1/8	2	29	0	0	2	10	
1/16	1	14	5	38	6	30	
1/32	1	14	4	31	5	25	0.159
1/64	2	29	1	8	3	15	
≥ 1/128	1	14	3	23	4	20	

\* < 0.05 = Sig.

\*\* < 0.001 = Highly sig.

ity = 92.9%, specificity = 100% and positive predictive value of 100% and negative predictive value of 85.7%. The diagnostic accuracy of the PCR genotyping is 95%. Contrasting results were obtained by Johnson et al. [30] for the exon 10 PCR. The sensitivity was higher, 94%, but the specificity was low, 36%.

The concordance rate between RhD genotypes from amniotic fluid and Rh D serotypes from cord blood was 95%. There were a highly significant correlation between the RhD genotyping and RhD phenotyping.

Fetal problems from Rh sensitization are detected with Doppler ultrasound testing and sometimes with amniocentesis. In response to an Rh-positive fetus, immune system may quickly develop IgG antibodies, which can cross the placenta and destroy fetal red blood cells. Each subsequent pregnancy with an Rh-positive fetus may produce more serious problems for the fetus. The resulting fetal disease (called Rh disease, hemolytic disease of the newborn, or erythroblastosis fetalis) can be mild to severe. Mild Rh disease involves limited destruction of fetal red blood cells, possibly resulting in mild fetal anemia [31]. The fetus can usually be carried to term and requires no special treatment. Moderate Rh disease involves the destruction of larger numbers of fetal red blood cells. The fetus may develop an enlarged liver and may become moderately anemic. The fetus may need to be delivered before term and may require a blood transfusion before (while in the uterus) or after birth. Severe Rh disease (fetal hydrops) involves widespread destruction of fetal red blood cells. The fetus develops severe anemia, liver and spleen enlargement, increased bilirubin levels, and fluid retention (edema). The fetus may need one or more blood transfusions before birth. A fetus with severe Rh disease who survives the pregnancy may need a blood exchange [32].

The present study demonstrated that fetal anemia was detected among RhD positive fetuses (the mean value of hemoglobin was  $10.6 \pm 4.7$  g%). As regards neonatal condition, seven RhD positive fetuses were admitted to NICU, four were born dead (IUFD), six fetuses had received postnatal treatment (phototherapy and exchange transfusion) and five fetuses had hydropic changes in ultrasound scan. All previous results implicate the correlation between the presence of RhD gene and the neonatal outcome. Our findings were in agreement with available data from Iran and other populations [33–35]. Also closely similar results were reported by Helen et al. [36]. A theoretical model indicated that amniocentesis with PCR-based techniques for fetal RhD typing would be associated with a fourfold reduction in perinatal loss compared with cordocentesis and serology for fetal typing.

The treatment of RhD alloimmunised pregnancies requires early and safe diagnosis of the fetal RhD type. The identification of an antigen-negative fetus on the basis of the blood group genotype provides significant advantages in managing the pregnancy at risk for HDFN.

## 5. Summary and conclusion

From the present study we can conclude that different options for management of Rhesus factor and D antigen (RhD) have been evolved alongside the technologic advances. The ability to determine the Rh status of the fetus early in pregnancy without invading the fetomaternal circulation and the use of

molecular biology techniques represents a major breakthrough in the clinical management of RhD alloimmunization.

## 6. Recommendations

- Routine screening should be done at the first prenatal visit to determine blood group, Rh status, and the presence of maternal antibodies.
- Use of different sets of gene specific primers at two different loci to improve the prediction of the genotype and to eliminate the possibility of genetic and laboratory sources of errors.
- Hydrops foetalis is a challenging entity for both the obstetrician and the neonatologist. Early diagnosis and treatment greatly improves perinatal outcome.

## Conflict of interest

The authors declare that there is no conflict of interest.

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