

The Noninvasive Fetal RHD Genotyping from Cell-Free Fetal DNA Circulating in Maternal Blood: A Feasible Tool in Clinical Practice of Mother-Fetus Rh Incompatibility in Romania

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Abstract

Introduction: The aim of this study was to develop a noninvasive method for fetal RHD genotyping by analyzing cell free fetal DNA circulating in the maternal blood in order to help improve the management of the mother-fetus Rh incompatibility. *Materials and Methods:* Total cell free DNA from maternal blood samples was extracted manually. In order to detect the targeted sequences, we develop two distinct end-point multiplex PCR reactions followed by automatic high-resolution capillary electrophoresis.

Results and Discussions: During our study, one hundred and thirty-six plasma samples withdrawn from women in their 5th to 28th weeks of pregnancy were tested. We evaluated the diagnostic accuracy of this noninvasive fetal RHD genotyping approach and found that we successfully genotyped one hundred and thirty-five (97.8%) samples. Three samples (2.2%) were inconclusive due to small amount of fetal DNA in maternal plasma, correlated with blood sampling too early in pregnancy (5th to 7th weeks). After the 9th week of pregnancy, we were able to retest only two of three samples, one sample being impossible to test because of a miscarriage. *Conclusion:* The results presented in this paper are demonstrating the feasibility of this protocol to correctly genotype the RHD gene and its potential value for the Romanian prenatal care, screening and diagnosis services.

Keywords: noninvasive, NIPD, cfDNA, RHD, alloimmunization, HDFN

1. Introduction

The blood groups analysis is mandatory in transfusion medicine. Beside the ABO blood system, the Rh blood group system is considered the most clinically significant in transfusion medicine [1 - 3]. Rh blood group system is highly polymorphic and immunogenic and the mother-fetus Rh incompatibility is the most common type of incompatibility mechanisms [2, 3]. Alloimmunization occurs in RhD negative pregnant woman, after a secondary exposure to D antigens of red blood cells (RBC) surface and is the most common cause of hemolytic disease of the fetus and the newborn (HDFN) [4]. In Caucasian population, about 10% of pregnancies are Rh incompatible [5] and the routine prenatal anti-D prophylaxis at 28-30 weeks of pregnancy is recommended in clinical practice, although about 38-40% of RhD negative women are carrying a negative fetus and receiving this blood product is unnecessary [4, 6 - 8]. The fetal RHD genotyping provides selection criteria for pregnancies requiring the anti-D administration [9]. The invasive determination of fetal RHD status can be a risky procedure because of the methodology of sampling the fetal cells [10]; the women with no RhD hemolytic

antibodies and with no recommendation for prenatal diagnosis of chromosomal abnormalities would be exposed to general risk associated with amniocentesis and CVS (chorionic villus sampling) [11]. Secondly, the already sensitized women could exhibit an increase severity of alloimmunization and fetal hemolysis [12]. The noninvasive approach for fetal RHD genotyping became the method of choice since the detection of cell free fetal DNA (cffDNA) originated from the placenta in the maternal circulation [13]. The cffDNA is detected starting with the 5th week of pregnancy [14, 15] and its quantity increases during pregnancy from 3.4% to 6.2% of the mean total DNA concentration in maternal plasma [15]. The short mean half-life of the cffDNA excludes the possibility of contamination from a previous pregnancy [16].

2. Materials and Methods

Patients

The local ethic commission approved this study. The participants for noninvasive prenatal diagnosis (NIPD) method validation were selected using the following criteria: (i) healthy individuals with no significant diseases in the past, and (ii) pregnant women previously tested for a positive ultrasound certifying singleton pregnancy. All the participants in this study were informed about this noninvasive approach for prenatal diagnosis and they signed an informed consent. The patients were classified in three major categories: non-pregnant female patients, male patients and pregnant RhD women having RhD positive partners. The majority of the pregnant women (76.4%) included in this study were also invasively investigated for chromosomal anomalies. All participants were tested serologically for reassurance of RhD status before the genetic test; the pregnant women were also tested to exclude the possibility for alloimmunization from previous events: pregnancy, abortion, blood transfusion. The subcategories are listed in Figure 1.

Sample preparation

Twelve milliliters of maternal blood were collected in EDTA vacutainers from each participant in this study. For pregnancies invasively tested for chromosomal anomalies, the venipuncture was performed before the invasive sampling. All samples were processed within 24 hours after venipuncture to prevent the excess lysis of maternal cells, a phenomenon followed by increased levels of cell-free maternal DNA. The first cell-plasma separation step was performed at a low speed centrifugation of 2200xg for 30 minutes (min). The plasma sample was carefully removed from the tube without disturbing the buffy coat, placed into a fresh tube, and centrifuged at 14000xg for 30 min; the supernatant was carefully transferred to a new tube and then stored at -80°C prior to DNA extraction.

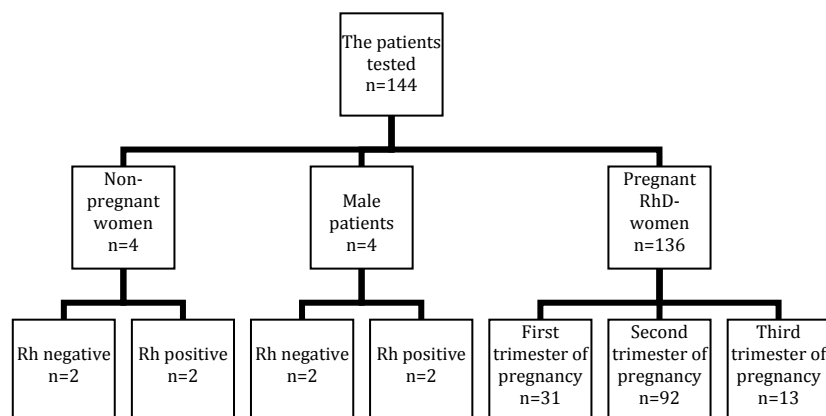


Figure1. Flow chart summarizing the patients included in this study

DNA extraction from maternal plasma

The plasma samples were gradually defrosted from -80°C to room temperature. Then, they were centrifuged at 14000xg for 10 min to eliminate any cryoprecipitate. The total cell-free DNA (cfDNA) extraction protocol was optimized starting from 1 mL of plasma. We used the QIAamp® DSP Virus kit (Qiagen) following the manufacturer protocol excepting that the reagents volume in the first part of the protocol was adjusted to the increased input of maternal plasma. In the DNA elution step, we used 45µl of elution buffer. Immediately afterwards we started target amplification. We introduced in each extraction procedure a non-template control sample containing ultra-pure water.

The multiplex PCR analysis

Two distinct multiplex PCR reactions were performed for the RHD genotyping using the Veriti® Thermal Cycler (Applied Biosystems). Considering the vast genetic diversity of the RHD gene, we chosen two targets, in order to avoid misleading results in cases with a partial D antigen [1, 17]. There were targeted two gene regions, from the fifth and respectively seventh exons, according to the recommendation of the Special Advanced in Fetal and Neonatal Evaluation (SAFE) European Community (EC)-funded network of excellence [18]. The exon 5's assay amplifies only the intact RHD gene; the exon 7's assay amplifies both RHD gene and RHD \square , the latter being common in Black Africans [17, 18]. RHD \square , also called silent RHD allele or pseudogene, is highly homologous with RHD, but is inactive [17]. RHD \square can exhibit different mutations such as a 37-bp duplication of the last 19 nucleotides of the third intron and the first 18 nucleotides of the forth exon [19] as well as a missense mutations in the fifth exon and a nonsense and missense mutation in the sixth exon [1]. Legler and coworkers [20] previously described the primers pairs for these two RHD gene specific sequences. We also included a reaction's internal control, the housekeeping gene β -globin [21]. The internal control allows PCR amplifications to occur from both fetal and maternal DNA and is considered as a control for the DNA extraction as well as for the amplification. The PCR reactions were performed using 1x Multiplex PCR Master Mix with HotStarTaq DNA Polymerase (Qiagen Multiplex PCR kit), and primers supplied by IDT, Inc (Table 1) and 10.7µl cell-free total DNA freshly extracted. The cycling conditions were 95°C for 15 min followed by 50 cycles of 95°C for 1 min, 60° C for 1 min and 72° C for 1 min.

Table 1. The primers sequences used for RHD genotyping and for the control marker

Primer name	Primer sequence (5'-3')	Conc (nM)	Amplicon size (bp)	References
<i>RHD ex7 fw</i>	GGG TGT TGT AAC CGA GTG CTG	400	125	Legler et al.
<i>RHD ex7 rew</i>	CCG GCT CCG ACG GTA TC	400		
<i>RHD ex5 fw</i>	CGC CCT CTT CTT GTG GAT G	400	82	Legler et al.; Finning et al.
<i>RHD ex5 rew</i>	GAA CAC GGC ATT CTT CCT TTC	400		
<i>β-globin fw</i>	CAA CTT CAT CCA CGT TCA CC	320	268	Bon et al.
<i>β-globin rew</i>	GAA GAG CCA AGG ACA GGT AC	320		

PCR multiplex reaction was optimized in order to eliminate the preferential amplification of one locus. To assess the reaction limit of detection we analyzed genomic DNA from non-pregnant RHD positive women. We performed a standard two-fold dilution series from 50 ng down to 6 pg of DNA, approximately equivalent to 1 genome (6.6 pg of DNA) [15, 22]. We correctly genotyped the RHD gene, also when starting from 6 pg of DNA. To test the reactions applicability to cell free DNA from plasma we tested both plasma samples from selected non-pregnant women and males: each group included two RhD positive and two RhD negative individuals. All the samples were amplified in triplicate (Figure 2). The PCR

products were analyzed automatically by high-resolution capillary electrophoresis on a QIAxcel system (Qiagen) using QIAxcel DNA High Resolution kit. The high-resolution gel cartridge allows the DNA fragments separation with a resolution of 3–5 bp and a detection sensitivity of 0.1ng/μl DNA from undiluted amplification reactions. This electrophoresis system offered a more robust result with less sample input material. DNA fragments size was measured by the inclusion of two DNA markers: QX DNA Alignment Marker 15 pb/1 kb (1.5 ml) and the QX DNA Size Marker pUC18/*Hae*III (Figure 2).

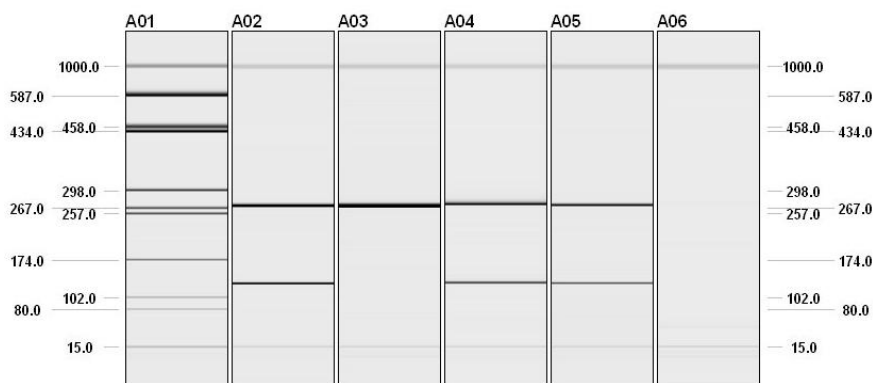


Figure 2. High-resolution capillary electrophoresis of exon 7 RHD reaction products - electropherogram view: A01- QX DNA Size Marker pUC 18/*Hae* III marker; A02 - RHD positive control: 125 bp amplicon for exon 7 and a 268 bp for β -globin; A03 - RHD negative control with a 268 bp amplicon for β -globin, A04 and A05 - RHD positive results; A06 - non-template control

All reactions were carefully monitored to prevent cross-contamination. The DNA extraction, the PCR reaction setup and the electrophoresis were performed in separate rooms and only aerosol-resistant pipette tips were used. There were included non-template controls in each step.

3. Results and Discussions

The results interpretation was made by applying a scoring model combining the results obtained for exon 5 and exon 7 in the RHD gene, and respectively for the β -globin sequence. Each run validation required a negative result for the extraction and PCR non-template controls. The samples amplification was considered valid if we obtained the β -globin specific amplicon (Figure 3). A RHD positive genotype is assessed when specific DNA bands are detected for both RHD sequences: exon 5 and exon 7. In cases where only exon 7 gives a positive signal we can suspect the presence of the pseudogene found especially in individuals of African descent [18, 19]. The negative result was considered when no RHD sequences specific amplicons were observed but only the positive amplification for β -globin. An inconclusive result was considered when either one of the two amplifications in RHD gene was negative. All three replicates were included in the analysis. A **final** positive result is validated when at least two out of the three replicates are positive; the result is inconclusive if just one is positive and the other two are negative. In the case of inconclusive results, we recommend repeating the test from another plasma aliquot. A negative result is considered when all three replicates are negative.

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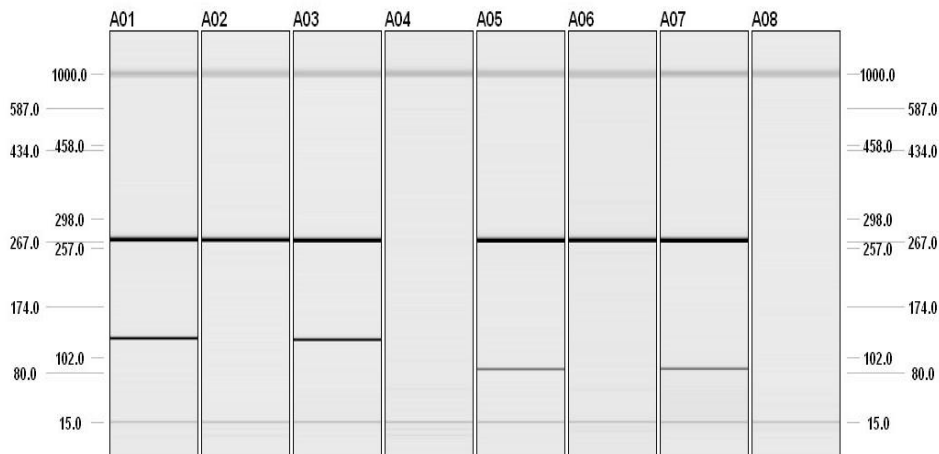


Figure 3. High-resolution capillary electrophoresis of RHD genotyping (exon 7 amplicon at 125 bp, exon 5 amplicon at 82 bp and β -globin amplicon at 268bp) - electropherogram view: A01 - exon 7 RHD positive control with positive β -globin; A02 - exon 7 RHD negative control with positive β -globin; A03 - exon 7 RHD positive sample with positive β -globin; A04 - exon 7 non-template control with negative β -globin; A05 - exon 5 RHD positive control with positive β -globin; A06 - exon 5 RHD negative control with positive β -globin; A07 - exon 5 RHD positive sample with positive β -globin; A08 - exon 5 non-template control with negative β -globin

We noninvasively analyzed 136 plasma samples from RhD negative pregnant women having RhD positive partners; from this pool, 104 women were also invasively tested for chromosomal abnormalities and we successfully confirmed the results of the noninvasive procedure (Figure 4).

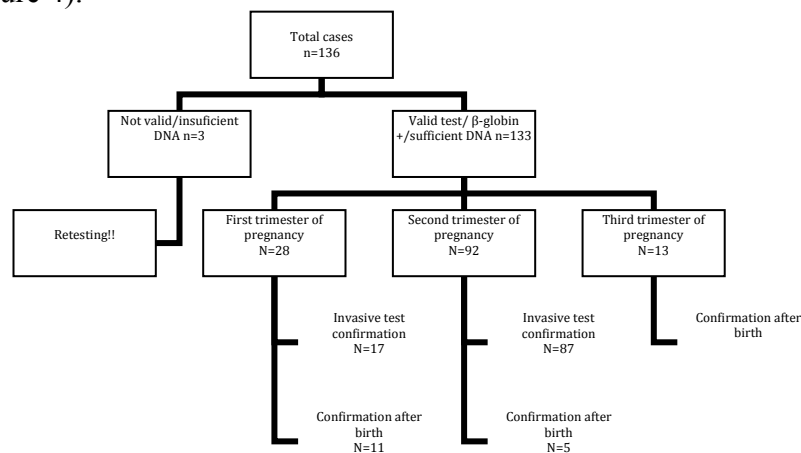


Figure 4. Flow chart summarizing the noninvasive detection of the RHD genotype in 136 RhD negative pregnant women

The remaining 29 cases were tested only by the noninvasive approach: 14 in the first trimester, 5 cases in the second trimester and respectively 13 in the third trimester. In three cases from early pregnancy (between the 5th and the 7th weeks) we obtained inconclusive results and recommended retesting after the 9th week of pregnancy. One woman miscarried shortly after the blood sampling therefore we could not confirm our result. According to the latest report of the Romanian National Institute of Statistics, the general population of our country exhibited a decreasing propensity of reproduction with a tendency to delay the birth of a first child [23]. Considering these facts, the national policy is towards the implementation of new strategies to increase the birth rate in conjunction with the prevention of pregnancy

anomalies associated with advanced maternal age [28]. Along with this prophylaxis approach, there are many safer diagnostic procedures for the fetus and for the mother. Therefore, this noninvasive approach for fetal RHD genotyping developed in our laboratory may offer the possibility to improve the Romanian prenatal care, screening and diagnosis services towards the mother-fetus Rh incompatibility. Our study focused on the possibility of testing of a rapid, efficient and less expensive noninvasive test for RHD genotyping in order to improve the management of non-sensitised RhD pregnancies in Romania. Another important advantage of this approach is the elimination of about 38-40% of the antenatal/post-delivery and post CVS/amniocentesis administration of anti-D vaccine because the fetus is not RhD-positive [4, 6 - 8]. We combined the end-point PCR along with the high-resolution electrophoresis and succeeded to detect a target concentration of 6pg as recommended by researchers in the noninvasive prenatal field [15, 22]; this DNA input is approximately equivalent with a genome copy and offered an assurance to proper testing [15]. In the process of choosing the suitable targets in the RHD gene, we followed the recommendation of the Special Non-Invasive Advances in fetal and Neonatal Evaluation Network of Excellence (SAFE NoE); it was stated that for the RHD correct genotyping should be used at least two sequences, preferably the exon 5 and the exon 7 [20]. The exon 7 is considered the most reliable from all ten exons in the RHD gene, although the exon 5 is to be used in order to detect the pseudogene RHD \square [11, 18]. We included in the RHD test an amplification internal control, the β -globin sequence, to confirm the DNA extraction and amplification and we evaluated the β -globin result versus RHD markers. Although in our study the majority of women tested were in the second and the third trimester of pregnancy, we were careful with the evaluation of the test accuracy especially for the first trimester of pregnancy because of the high probability of insufficient fetal DNA in maternal blood circulation [13 - 16]. This evaluation was focused on the first trimester testing in order to decide the safety cut-off for test recommendation. We analyzed 31 samples from pregnancies between 5 and 12 weeks old. Three samples from 5th, 6th and 7th week of pregnancy had inconclusive results (Table 2). The reason behind these inconclusive results was the insufficient fetal DNA in the sample, as previously reported by others [13 - 16, 24]. In order to evaluate these three findings we eventually analyzed two commonly used sequences on the Y chromosome: the single copy SRY gene and the multi-copy sequence DYS14 as described previously [25]. Two samples, taken from the 5th and 7th weeks of pregnancy were negative for both SRY and DYS14 markers. The GAPDH control marker showed a positive weak signal that could be interpreted as female sex, but considering the early pregnancy, we were not very confident with this report. For the third sample collected at the 6th week of pregnancy, we detected a negative signal for SRY, a positive signal for DYS14 and a low internal control. These findings indicate a male characteristic result, but the result could not be reported according to the noninvasive gender determination protocol [25] (Table 2). The positive amplification of the multi-locus DYS14 marker and the negative result for SRY single locus marker confirmed our hypotheses that there was a small amount of fetal DNA in the maternal sample. We excluded the cross-contamination as all the controls were as expected and we confirmed our result by retesting the samples at 10 weeks of pregnancy. All three women were kindly asked to come for retesting at 9-10 week of pregnancy as recommended by others [13 - 16].

Table 2. Evaluation of the inconclusive results of the RHD genotyping assay

Sample no.	Week of pregnancy	RHD gene Exon 5	RHD gene Exon 7	Beta globin	SRY	DYS14	GAPDH
1	5 th	-	-	+	-	-	+
2	6 th	-	+	+	-	+	+
3	7 th	-	-	+	-	-	+

Although the 8th week of pregnancy is considered as a good cutoff threshold for blood sampling in noninvasive testing [13, 15, 16, 26], the current recommendation for blood sampling in noninvasive testing is 11 weeks. This recommendation is in accordance with safety measures in order to minimize the false-negative results and to coincide with routine antenatal visits [8]. Patient no.1 miscarried and retesting was not feasible. The patient no. 2 received a RHD positive result and a male gender result after retesting at 9th week of pregnancy and the patient no. 3 received a RHD negative genotype and a female gender result at 9 weeks and 6 days of pregnancy. The other 28 samples were collected between 9 to 12 weeks of pregnancy and a RHD genotype was reported for each one. Based on these results the overall test accuracy was 97%. For the first trimester of pregnancy, the accuracy of this noninvasive testing was 90%, smaller comparing with the overall testing because of the biology of the fetal DNA in maternal circulation [27].

3. Conclusions

Using two different sequences in the RHD gene, three replicates for each reaction and a specific scoring model for results interpretation, led us to a the correct evaluation of noninvasive fetal RHD genotyping. These results confirm the utility for the Romanian prenatal care, screening and diagnosis services.

4. Acknowledgements

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