

# FETAL RHD GENOTYPING FROM MATERNAL PLASMA IN A POPULATION WITH A HIGHLY DIVERSE ETHNIC BACKGROUND

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## SUMMARY

**OBJECTIVE.** To establish the performance of conventional PCR as a noninvasive method for fetal genotyping, by free fetal DNA analysis of distinct RHD regions from maternal plasma, in a population of a diverse ethnic origin.

**METHODS.** We conducted a validity of the diagnostic test by analyzing 81 plasma samples from RhD-negative Brazilian pregnant women, from 4 to 41 gestational weeks. We tested for exon 10 and intron 4 gene regions by allele specific-PCR. Fetal RHD genotyping by PCR on maternal plasma was compared to serologic RhD typing in the neonatal period.

**RESULTS.** Samples were obtained as follows: 15 in the 1<sup>st</sup>, 37 in the 2<sup>nd</sup> and 29 in the 3<sup>rd</sup> trimester. General accuracy was 97.3%, sensitivity of 98.3% and specificity of 93.8%.

**CONCLUSION.** Conventional PCR is an accurate method for fetal RHD genotyping on maternal plasma, even in a population of mixed ethnic origin.

KEY WORDS: Free fetal DNA. PCR, RHD genotyping. Maternal plasma. Prenatal diagnosis.

## INTRODUCTION

Hemolytic Disease of the Fetus and the Newborn (HDFN) still contributes to perinatal morbidity and mortality, in spite of the widespread immunoprophylaxis<sup>1,2</sup>. Currently, the main problem in the management of RhD-negative women is how to avoid invasive investigations for as long as possible. The Rh antigens are the main cause of HDFN. Because of its strong immunogenicity, RhD is the most important antigen of the polymorphic Rh system.

In the management of pregnancies, prenatal determination of fetal RHD genotype can be useful. When the father is heterozygous at the RHD locus, the fetus has a 50% chance of being RhD-negative and for this reason unaffected. In an immunized mother when the fetus is predicted to be RHD-negative, the need for further invasive techniques is reduced. Therefore, transplacental hemorrhage with deterioration of clinical features and fetal losses related to procedures are avoided. Furthermore, additional investigations and unnecessary immune globulin anti-D administration are avoided in non-immunized pregnant women.

To analyze fetal DNA, many polymerase chain reaction (PCR)-based tests have been reported, using invasive procedures<sup>3,4,5</sup>. The recent identification of high concentrations of cell-free fetal DNA in maternal plasma<sup>6</sup> has made noninvasive fetal RHD genotyping possible. It has been demonstrated that real-time PCR assays can provide accurate results in a quick and non-demanding way<sup>7-12</sup>. Unfortunately, performance of the fluorescence-based real-time polymerase chain reaction is restricted to a few specialized laboratories. In recent studies, conventional PCR assays have been used for this purpose<sup>13-16</sup>.

Most of the primer sets developed for molecular fetal genotyping were based on sequences of individuals usually of Caucasian origin. It is now known that there are genetic causes for the RhD-negative phenotype of an individual with an incidence that varies according to ethnic origin. The Brazilian population is of heterogeneous ethnical origin and is unevenly distributed within this country of continental dimensions. The intense process of miscegenation made the Brazilian population unique in its ethnic background<sup>17</sup> without any clear distinction of races in the country.

That is why we conducted a validity study to evaluate the clinical application of fetal RHD genotyping from maternal plasma using the conventional PCR, by assessing its performance in a population with a varied ethnic origin.

## METHODS

Subjects and blood samples: Peripheral blood samples (5 – 10 ml) were obtained from 81 RhD-negative pregnant women, with singleton pregnancies, during a routine prenatal visit. The subjects were from all regions of Campinas, a city in the Southeast of Brazil. This population was of a highly mixed ethnic background, with an admixture between descendants of Europeans, Africans and indigenous people. Blood samples from sensitized women were collected prior to invasive procedures. All women gave informed consent. Approval for the study was provided by the Institution's ethical committee.

All samples were processed and analyzed at the laboratory of molecular biology for blood groups at the Hemocentro Unicamp (UNICAMP - Campinas - Brazil). The samples were numbered according to the time of collection.

Samples were collected into vacutainer blood tubes containing EDTA and centrifuged at 3000g for 20 minutes to separate the plasma. Plasma aliquots were stored at 4 °C until further processing. Before DNA extraction, each plasma sample was re-centrifuged at 3000g for 15 minutes and the upper phase was used for the DNA extraction.

To confirm the gestational age, all women were submitted to an ultrasound evaluation.

**DNA extraction:** DNA was extracted from 500 ml of the plasma sample, using the Easy DNA Kit (Invitrogen®, Carlsbad, CA) according to the manufacturer's recommendation. Ten ml of the DNA were used for the PCR assay.

**PCR analysis:** A Perkin Elmer thermal cycler (model 9700, Foster City, CA) was used for the PCR. The primers and amplification conditions used for RHD genotyping have been previously published<sup>18</sup>. The PCR was formed with 50pmol of each primer, 2nmol of each dNTP, 1.0 U of Taq DNA Polymerase (Invitrogen®, Carlsbad, CA) and buffer in a final volume of 50ml.

Negative internal water control was included to check possible contamination. To ensure quality control of the reagents, control reactions containing known RhD-positive and RhD-negative DNA were performed simultaneously with the test reaction.

Thermal cycling was carried out in 35 cycles after 15 minutes of denaturation at 95 °C. Each cycle consisted of: 94 °C for 20 seconds, 62 °C for 20 seconds, 72 °C for 20 seconds and 72 °C for 10 minutes. Five to 10 ml of each DNA product were size-separated by electrophoresis in 1–2 % agarose gel for 45–60 minutes at 100 V, visualized by ethidium bromide (0.5ml) staining and photographed under ultraviolet light to confirm amplification.

**RHD genotyping:** The PCR products examined in the agarose gel were interpreted based upon the differences between RHD and RHCE genes in two genomic regions: intron 4 and exon 10. For intron 4, a set of 3 primers (RH141, RH142, RH143) yielded a product of 115 bp for RHD and 236 bp for RHCE. For exon 10, a common 5' primer (EX10F) was used for both RHD and RHCE. When paired with the RHD-specific 3'-untranslated region primer (RHD3'-UTR), it yielded a product of 245 bp, and when paired with the RHCE-specific-3' – UTR primer (RHCE3'-UTR), it yielded a product of 160 bp. The fetus was predicted to be RhD-positive when either the RHD exon 10 or the intron 4 were amplified, and to be RhD-negative when no RHD signal (nor RHD intron 4 neither exon 10) was obtained and at least one region of the RHCE gene was amplified.

At the time of analysis, the investigator performing the PCR did not know the RhD type of the fetus.

**Anticontamination measures:** To prevent contamination of the PCR assays separate areas were set up for each step described above, in addition to the internal water control of the PCR reaction mixtures. All manipulations were carried out with disposable material and frequent changes of gloves.

**Neonatal RhD phenotyping:** Umbilical cord blood samples from neonates or fetuses were collected into EDTA tubes and phenotyping was determined by hemmagglutination in gel cards (Diamed AG, Morat, Switzerland). Samples from neonates were collected during delivery.

**Statistical analysis:** The sensitivity, specificity, positive and negative predictive values were tested by comparing the PCR results with RhD serologic typing in neonatal blood. For all statistical procedures, the SAS – 8.2 version Ó (1999–2001) software was used.

**Table 1 – Fetal RHD genotyping from the maternal plasma by allele-specific PCR at different trimesters of gestation (N=75)**

Trimester	Number	RhD-positive fetuses		RhD-negative fetuses	
		PCRRHD-pos	PCRRHD-neg	PCRRHD-pos	PCRRHD-neg
First	15	10	1	-	4
Second	34	29	-	-	5
Third	26	19	-	1	6
<b>Total</b>	<b>75</b>	<b>58</b>	<b>1</b>	<b>1</b>	<b>15</b>

## RESULTS

A total of 81 maternal plasma samples were analyzed by this PCR approach. The gestational ages at the time of blood sampling ranged from 4 to 41 weeks (mean: 24 weeks, median: 25 weeks). Fifteen samples were from the first trimester (18.5%), 37 from the second (45.7%), and 29 from the third trimester of gestation (35.8%). RhD type of newborn blood was obtained from all samples. Among the 81 newborns, 62 were RhD-positive and 19 were RhD-negative.

Amplification of fetal DNA (RHD/CE) was successful in 75 of 81 fetuses (92.6%). In the six remaining cases, despite repeated attempts, neither the RHD nor the RHCE genes were identified. Three of these six non-interpretable results were RhD-positive on post-delivery phenotyping and they were from the second (1 case) and third (2 cases) trimesters. The other three samples were from RhD-negative fetuses, 2 from the second trimester and 1 from the third.

Of the 75 samples that were successfully amplified, 59 were RHD-positive and 16 were RHD-negative. One RhD-positive fetus from the first trimester (12 weeks) was genotyped as RHD-negative (false negative), and one RhD-negative fetus from the third trimester (34 weeks) was genotyped as RHD-positive (false positive). Repeated PCR analysis of these misgenotyped samples confirmed the false results. It was not possible to resample the discordant individuals and to genotype the family.

Results of the RHD-PCR assay of maternal plasma samples are shown in TABLE 1. The overall sensitivity of determination of RHD fetus status from maternal plasma was 98.3% and the specificity was 93.8%. A concordance rate of 97.3% (73/75) was observed between polymerase chain reaction and serologic typing. The earliest positive result obtained was at 4 weeks of gestation.

## DISCUSSION/CONCLUSION

This prospective study demonstrated that the conventional polymerase chain reaction analysis of the maternal plasma can be used for the fetal RHD genotyping, even in a miscellaneous ethnic population. Although there have been several reports on maternal plasma analysis for determination of the fetal RHD status, this study has the largest number of cases using conventional PCR and is the first with a Brazilian population sample.

The selection of a specific DNA typing system may be oriented by practical considerations, specific for a particular laboratory and population. It is therefore essential that more studies to assess the

validity of any RHD genotyping by PCR for a particular population be carried out. The Brazilian population has unique genetic diversity and the women under study could not be classified as true Caucasian or Black. Rules applied for interpretation of genotype in populations of a clear genetic background are not applicable to this population and to those with similar characteristics.

Individual laboratories should assess their own level of accuracy to ensure a better management approach for HDFN. A total concordance rate of 97.3% was observed in the Hemocentro Unicamp laboratory. A similar concordance rate of 97.8% was observed by Johnson et al.<sup>15</sup> using multiple exons (exon 4, 5 and 10) in a conventional multiplex PCR. Other conventional PCR reached 100% of concordance as in Faas et al.<sup>13</sup> using exon 7, and Nelson et al.<sup>14</sup> using exon 10 in 26 RhD-positive fetuses. In contrast, Siva et al.<sup>16</sup>, amplifying exon 7 and exon 10, reached 80.8% of concordant results in 26 maternal samples.

The conventional PCR can easily be performed in any laboratory using basic molecular biology techniques. It is a simple, accurate, rapid, non-radioactive, and inexpensive DNA-based method. Bischoff et al.<sup>19</sup> compared RHD/CE-PCR results between conventional and real-time methods, in plasma samples of 20 mothers bearing RhD-positive fetuses, amplifying exon 7 of both genes and encountered a sensitivity of 62% with the real-time and only of 50% with the conventional PCR. In this study a sensitivity of 98.3%, was reached, probably because two sets of primers were used.

There are some technical difficulties associated with the PCR, for example the rate of amplification failure. In this study a rate of 7.4% (6/81) was encountered, similar to Siva et al.<sup>16</sup> who used the conventional PCR to amplify exon 7 and exon 10 of the RHD and RHCE genes and were unable to amplify 7.1% of 28 maternal plasma samples (2/28). The same 7% rate of amplification failure was found by Smid et al.<sup>20</sup>, using the conventional Y-PCR for sexing 27 fetuses (2/27). Lower, but not absent, rates were observed by using the real-time PCR. Legler et al.<sup>10</sup> reached a 2.8% of unsatisfactory amplification (1/36) and Randem et al.<sup>11</sup> reported a 5.3% rate (6/114).

The lack of efficiency on detection probably reflects variable fetal DNA concentration in maternal plasma and could be due to DNA degradation from storage conditions or instability of the PCR products<sup>21</sup>. In the current study it is possible that storage conditions and time to prepare the samples may have affected the quality and the quantity of fetally-derived DNA.

Every effort should be made to avoid incorrect typing of the fetuses. Chan et al.<sup>22</sup> advocate the use of several primers in separate reactions, in addition to performing complete family analysis in cases of Rh alloimmunized pregnancies. Van den Veyver and Moise Jr<sup>23</sup> recommend the same family study and the use of two sets of primers, however in a single reaction. In this study two regions of the RHD gene were used to partially eliminate the false-negative results, but to date, it is still not possible to avoid false-positive results. The inability to reassess the fetal and patient's family for new serologic type and genotyping is a fully recognized limitation of this study.

For the false positive result obtained in the present study, only the RHD exon 10 was amplified. According to Finning et al.<sup>24</sup>, as this region of RHD is present in both RHDy and RHD, when just the exon 10 is amplified and no other RHD signals are obtained, the fetus should be predicted to be RhD-negative. So, the current false-positive result in

question could be explained by this intact but nonfunctional RHD (RHDY) which has a high frequency among Africans<sup>25</sup>. Some authors indicate that all RHD genotyping in non-Caucasians individuals should take in account the RHDY<sup>26-28</sup>. The Brazilian population is predominantly from non-Caucasian origin. The potential "D variant", cause of false-positive result, was not examined here using the present method. Aware of the extreme sensitivity of PCR, it was also not possible to exclude PCR contamination, notwithstanding the exhaustive steps of anti-contamination measures employed here.

False-negative results may be clinically more serious, leading to a delay or omission of intrauterine therapy. The herein false negative case might be explained by the presence of possible hybrid genes (RHD-CE-D<sup>s</sup>) because just the exon 10 RHCE gene region was amplified. It is important to note that the presence of fetal DNA in every sample cannot be confirmed and an ideal internal control to confirm the presence of fetal DNA is not yet available. Therefore when an RHD signal is not detected the possibility remains that this is due to lack of fetal DNA. The amount of cell-free fetal DNA is obviously a limiting factor for fetal RHD genotyping, especially during the first trimester of gestation. This might also explain the false-negative result in this study.

Unquestionably for fetal genotyping, the maternal plasma-based noninvasive method has considerable merit and will soon become more widespread.

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## No conflicts of interest

## RESUMO

### GENOTIPAGEM RHD FETAL POR MEIO DO PLASMA MATERNO EM UMA POPULAÇÃO DE ALTA DIVERSIDADE ÉTNICA

**OBJETIVO.** Avaliar o desempenho da reação em cadeia da polimerase (PCR) em gel (convencional) como método diagnóstico não-invasivo para a genotipagem RHD fetal, por meio da análise do plasma materno.

**MÉTODOS.** Foi conduzido um estudo de validação de teste diagnóstico a partir de 81 amostras sanguíneas obtidas de gestantes brasileiras RhD-negativo, entre 4 e 41 semanas de gestação. As regiões exon 10 e intron 4 do gene RHD foram testadas por meio da reação em cadeia da polimerase alelo-específica (AS-PCR) convencional. Os resultados da genotipagem fetal foram comparados com a tipagem sanguínea convencional no período neonatal.

**RESULTADOS.** Quinze amostras foram obtidas no primeiro trimestre, 37 no segundo trimestre e 29 no terceiro trimestre. Houve falha de amplificação em 6 amostras. A concordância entre os resultados da genotipagem e da tipagem neonatal foi de 97,3%, sensibilidade de 98,3% e especificidade de 93,8%.

**CONCLUSÃO.** AS-PCR convencional é um método com bom desempenho para a genotipagem RHD fetal por meio da análise do plasma materno, mesmo em uma população com alto grau de miscigenação. [Rev Assoc Med Bras 2006; 52(4): 232-5]

UNITERMOS: DNA fetal livre. PCR, genotipagem RHD. Plasma materno. Diagnóstico pré-natal.

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