Fetal *RhD* genotyping by real time quantitative PCR in maternal plasma of RhD-negative pregnant women from the Sahel of Tunisia

**Abstract.** *Objective:* The aim of this study was to evaluate the diagnostic value of *RhD* fetal genotyping from the plasma of RhD-negative pregnant women. *Methods:* We analysed the plasma samples of 65 pregnant women. DNA quantification was done using real time quantitative PCR (RQ-PCR) in multiplex targeting multiple *RhD* exons 5, 7 and 10, with a standardized pool of plasmid calibrators. Results were compared with serological analysis of cord blood after delivery. *Results:* Fetal *RhD* status was predicted with 95.38% accuracy from maternal plasma of pregnant women in the 11th to 40th weeks of gestation. One false positive but no false negative results were found. Thus the sensitivity of the assay was 100% and the specificity was 94.44%. *Conclusion:* The present data demonstrates that the fetal *RhD* genotyping approach could be achieved efficiently with RQ-PCR for RhD-negative tunisian pregnant women.

**Key words:** maternal plasma, fetal DNA, cell-free DNA, *RhD* gene, real-time quantitative PCR, *RhD* genotyping

Résultats. *Objectif:* évaluer la valeur diagnostique de l’approche du génotypage rhesus fœtal à partir du plasma maternel dans une population de femmes enceintes RhD-négatif. *Patientes et méthodes:* les prélèvements plasmatiques des 65 femmes enceintes ont été analysés. L’ADN a été quantifié par PCR quantitative en temps réel (RQ-PCR) par multiplexage ciblant les exons 5, 7 et 10 du gène *RhD*, et à l’aide d’une gamme de calibration plasmidique standard. Les résultats ont été comparés avec ceux de l’analyse sérologique du sang du cordon suite à l’accouchement. *Résultats:* le statut *RhD* fœtal a été prédit avec une précision de 95,38 % à partir du plasma maternel des femmes enceintes entre la 11e et la 40e semaine d’aménorrhée. Un faux positif, mais aucun faux négatif n’a été identifié. La sensibilité et la spécificité du test sont respectivement de 100 % et 94,44 %. *Conclusion:* ces résultats démontrent que l’approche de génotypage rhesus fœtal pourrait être efficace par PCR quantitative en temps réel chez les femmes enceintes tunisiennes de phénotype RhD négatif.

**Mots clés:** plasma maternel, ADN fœtal, ADN des cellules libres, gène *RhD*, PCR quantitative en temps réel, génotypage RhD
Rhesus (Rh) blood group incompatibility between the pregnant women and her fetus is a significant problem due to the possibility of maternal allo-immunisation and consequent haemolytic disease of the newborn (HDN) [1]. In Tunisia, HDN due to RhD immunisation is currently prevented in the vast majority of cases by administration of anti-D immunoglobulin to D-negative women within 72 h of each delivery of a RhD-positive newborn as well as in abortion cases. A dose of 300 μg is commonly used and this can be increased in case of feto-maternal haemorrhage.

A systematic antenatal prophylaxis that could allow the strengthening of prevention for all pregnant women is not recommended in Tunisia since such proceedings would expose to a potential shortage in anti-D immunoglobulin and a significant potential cost. Therefore antenatal prophylaxis is applied only in cases with obstetrical problems (amniocentesis and every cause of feto-maternal haemorrhage). Despite the previous precautions of Rh immunoglobulin prophylaxis in RhD-negative pregnant women, residual cases of Rh immunisations still occurs [2], unfortunately, the frequency of fetal immunization in Tunisia has not been determined, studies performed in France suggest that it would represent 0.9/1000 births [3-5]. The recent discovery of cell free fetal DNA in maternal peripheral blood (serum or plasma) in relatively high amounts compared with the cellular fraction [6]: 3.4 and 6.2% in early and late pregnancy respectively [5, 7] opened new possibilities for non-invasive prenatal diagnosis [8].

Using real time PCR technology, it is possible to successfully determine the RhD status of the fetus based on the assumption that the RhD gene is absent in RhD-negative pregnant women. Although, RhD gene deletion is the most common cause of the RhD-negative phenotype in the Caucasian population, RhD-negative phenotype in the black African population is commonly caused by the presence of a RHDψ with the frequency of 67% [9] and the d(C)ce haplotype with the frequency of 15% [10]. In Tunisia, a recent study revealed that the RhD-negative phenotype was highly associated with a deletion of the RhD gene in 99.32% of cases, nevertheless, there was a low African contribution associated to the presence of RHDψ (0.23%) and d(C)cehaplotype with the frequency of 0.46% which is considerably low compared to African black groups [11]. As these phenotypes could generate “false-positive” results in RhD genotyping assays, a genotyping strategy with cell free fetal DNA should be more specific (amplification of the desired DNA sequence only) and sensitive (able to detect a very low DNA copy number) than a strategy developed for use on genomic DNA.

Therefore, in the current study, we evaluate the diagnosis value of RhD fetal genotyping using a novel approach that consist to quantify fetal DNA level in maternal peripheral blood using the real time quantitative PCR targeting multiple exons of RhD gene with a standardized pool of plasmid calibrators.

**Material and methods**

**Maternal blood samples**

10 mL of EDTA anticoagulated blood samples were collected from 65 pregnant women recruited with informed consent at the Department of obstetrics and gynecology, Farhat Hached hospital and at the Center of maternal and child prevention of Sousse. The study was approved by the committee of ethics and research for the university hospital Farhat Hached of Sousse. Samples were obtained at a gestational stage ranging from 11 to 40 weeks. The RhD status of the fetuses was confirmed at the time of birth by standard serology protocols on cord blood cells. Blood samples were shipped to the laboratory within 2 hours to 3 days at room temperature and directly centrifuged two times at 3,000 g for 10 min. Supernatants were aliquoted and stored at -20°C until further processing.

**Plasma DNA extraction**

DNA was extracted from 200 μL of plasma sample with the biorobot easyMAG (Biomérieux) using the specific protocol according to the manufacturer’s instructions. For a control of the fetal DNA isolation, each run included a plasma pool of RhD-negative women pregnant with a RhD-positive fetus and a plasma pool of D-negative women pregnant with a RhD-fetus. The isolated DNA was eluted in 70 μL.

**RQ-PCR analysis**

The DNA was quantified by real time quantitative (RQ)-PCR in multiplex targeting RhD exon 5, RhD exon 7, RhD exon 10 and β-globin gene.

RQ-PCR analysis was performed using Abi-Prism 7900 Sequence Detection (Applied Biosystems, France). The RhD exon 10 TaqMan system consisted of forward primer, 5’- CCT CTC ACT GTT GCC TGC ATT-3’; reverse primer 5’- AGT GCC TGC AAC ATT-3’ and a probe 5’-(VIC) TAC GTG AGA AAC GCT CAT GAC AGC AAA GTC T (TAMRA)-3’ [12]. The RhD exon 7 TaqMan system consisted of forward primer 5’- GGG TGT TGT AAC CTA TCA TGG GCT ACA A (TAMRA)-3’; reverse primer, 5’- CCG GCT CCG ACG GTA TC-3’ and a probe 5’-(FAM) CCC ACA GCT CCA TCA TGG GCT GTA A (TAMRA)-3’ [12]. The RhD exon 5 TaqMan system consisted of forward primer 5’- CGC CCT CTG GTG GAT G-3’; reverse primer, 5’- GAA CAC GGC ATT CTT CCT TTC-3’ and a probe 5’- (VIC) TCT GGC CAA GTT TCA ACT CTG CTC TGC
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RQ-PCR was performed in multiplex amplifying all analysed samples. The Taq Man Universal PCR master mix (Applied Biosystems). The Taq Man amplifications reactions were set up in a reaction volume of 25 μL separately in two tubes. Taq Man amplifications reactions were set up in a reaction volume of 25 μL using the Taq Man Universal PCR master mix (Applied Biosystems). The RhD and β-globin probes (Fam-labeled probe β-globin, VIC-labeled probe RhD exon 10, Fam-labeled probe RhD exon 7 and VIC labeled probe RhD exon 5 were used at a concentration of 100nM; the PCR primers were used at a concentration of 300 nM. All reactions were run with a template volume of 6 μL. Amplifications were carried out in duplicate in 96 wells reaction plates. The TaqMan PCR conditions consisted in a first step at 50°C for 2 min, followed by denaturation at 95°C for 10 min and then 50 cycles at 95°C for 15 s and 60°C for 1 min.

β-globin plasmid calibrator purchased from Ipsogen (Marseille, France) was used to quantify the total amount of DNA (maternal DNA and fetal DNA) for RhD exons 5,7 and 10 quantification plasmid calibrators were developed and produced in the laboratory of molecular biology oh Hospital Nord (Marseille, France). The standard curves consisted on serial dilution of mixed plasmids (β-globin + RhD exon 10) and (RhD exon 7 +RhD exon 5) with a range of 5 to 50,000 copies/ 6 μL. PCR efficiency was evaluated from these standard curves for each target.

In the RQ-PCR approach, the liberation of a fluorescent reporter, tagged onto a sequence specific probe, is coupled to the amplification reaction. The sequence detector monitors the increase in fluorescent signal of each reaction well and determines the number of amplification cycles required to reach a fixed threshold signal intensity, termed the threshold cycle (Ct). The threshold chosen for the Ct calculation was 0.03. Background signal was under the threshold. The standard curve of plasmid calibrators generated a mean slope of ~−3.3±0.3 for both RhD and β-globin plasmids. Amplification results were reported by cycle threshold (Ct), that is the calculated cycle number at which the PCR products crossed a detection threshold and then results were expressed in terms of copies per milliliter (copies/mL) [8].

**Interpretation criteria**

The amount of maternal DNA was considered suitable for analysis when β-globin copies were comprised between 1,700/mL and 100,000/mL. A fetus was characterized as RhD-positive if at least one of two replicates was positive for all three RhD exons. When there were discrepancies between the three RhD exons, RQ-PCR assays targeting the exons with negative results were repeated on two additional replicates prepared from a new extract of the last preserved maternal plasma sample. If the discrepancy persisted, the result was considered inconclusive and the sample was excluded assuming that problems with sample storage or extraction of fetal DNA would compromise accuracy and reproducibility of the analysis test.

**RhD phenotyping on cord red blood cells**

The RhD phenotype was determined on blood samples collected at birth for Rh immunoprophylaxis. RhD serotyping was performed with commercial anti-D reagents (Dia-gast, France) testing the following specificities (P3X61; P3X21223B10; P3X290; P3X35) using a direct agglutination test. Indirect antiglobulin test was performed systematically for RhD-negative results.

**Contributions**

This study was done between two distinct groups; sample collection, plasma preparation and RhD phenotyping was performed in blood transfusion center of Sousse in Tunisia. Fetal DNA extraction and RhD fetal genotyping were performed in the laboratory of biochemistry and molecular biology in the hospital Nord of Marseille in France where the method has been developed.

**Results**

**Accuracy of fetal RhD genotyping from maternal plasma**

The RhD status of the fetus in 65 pregnancies was analyzed by RQ-PCR using fetal DNA derived from maternal plasma. Fetal RhD status was predicted in 63 pregnant women, 2 plasma samples were undetermined because of an excess of maternal DNA indicated by a high concentration of β-globin copies (> 100,000 copies/mL). Non-invasive prenatal fetal RhD exon 10, exon 5 and exon 7 genotyping analysis of maternal plasma samples was in complete concordance with the analysis of cord blood in 62 RhD-negative pregnant women delivering 45 RhD-positive and 17 RhD-negative newborns. One maternal plasma derived DNA, was predicted to be RhD-positive but at birth the baby was serologically RhD-negative, this discordance being most likely explained by the presence of an RhD polymorphism (weak D or partial D). Unfortunately, no cord blood was available to perform
an RhD genotyping and to validate this hypothesis. However, the positive and the negative predictive value of the fetal RhD status were 97.83% and 100% respectively.

The quantity of total DNA and fetal DNA in maternal plasma

The quantity of maternal DNA and fetal DNA in maternal plasma were investigated in 63 pregnancies. The amplification efficiencies of the assays specific for RhD exons 5, 7 and 10 were comparable, as indicated by similar slope values.

Plasma samples from pregnant women carrying a RhD-positive fetus gave Ct values in the range 33-40 according to the gestational age of pregnancy, whereas no Ct values were observed when the fetus was RhD-negative. When positive signals were obtained, the Ct values were used to predict the quantity of fetal and total plasma DNA from a standard curve calculated from known quantities of input DNA. Our data, therefore, report the mean number of copies of fetal DNA detected in maternal plasma, 17 maternal plasma samples were uninformative because no fetal DNA (RhD gene) was detected. The fetus was subsequently, confirmed to be RhD-negative in these pregnancies. The quantity of total DNA (β-globin) in maternal plasma was found to vary considerably (2667.21 copies/mL to 95405.16 copies/mL; mean: 13652.26 copies/mL). The mean number of copies of fetal DNA present in maternal plasma was found to increase with each trimester (table 1).

Representative amplification data of the RQ-PCR of 2 plasma samples: one predicted to be RhD-positive and another one predicted to be RhD-negative were shown in figure 1.

Discussion

Non-invasive prenatal diagnosis is one of the many major goals in human genetics. The discovery of cell free fetal DNA in maternal plasma in 1997 [6] opened new possibilities for non-invasive prenatal diagnosis. Circulating fetal DNA has been shown to increase in concentrations with gestational age and to be cleared rapidly following delivery [6, 11]. With the use of RQ-PCR methodology, circulating fetal DNA has been detected robustly in the plasma of pregnant women, even early in the first trimester of pregnancy [14]. In the present study, we performed a fetal RhD genotyping using maternal plasma from 65 pregnant women in weeks 11-40 of pregnancy. Postpartum serological RhD typing of the babies showed an overall discrepancy between genomic and serological typing of 1.53% related to one false positive result encountered in this study. In this case, RhD exon 5, 7 and 10 were detected in maternal plasma with ratio (number of fetal DNA copies/number of maternal DNA copies) below 6% which represent obviously fetal DNA. This pattern suggests the presence of paternally inherited weak or partial D allele taking account the high polymorphism of the Rh system. Such RhD variant was previously found in Tunisian blood donors in an independent study [11]. Contamination is not unlikely as the negative control was always negative. Unfortunately, this suspected result could not be ascertained since no infant blood was available, but this false positive is surely lesser worry than false negatives since the consequence of a false positive RhD determination for the fetus, would result only in unnecessary prophylaxis. Two pregnant women could not be evaluated for prenatal RhD typing, in the current assay, because of an excess of maternal DNA detected in the plasma which compromising the sensitivity of the test to detect fetal DNA. Excess maternal DNA could arise from cellular lysis before the plasma is removed. This was probably due to variation in time spent in transit (2 hours to 3 days). Furthermore, it has been reported that significant random variation can be found in the amount of total DNA, thus samples of maternal blood processed a long transit time (more than 2 days) after maternal venipuncture would yield a high amount of free maternal DNA [15].

Therefore, despite the previous cases, a reliable fetal RhD genotype determination was highly accurate in the present study. The positive and the negative predictive value of the fetal RhD status were 97.83% and 100% respectively, results were in complete concordance with that obtained by serologic study of the newborn for 62 pregnant women (95.38%). Accordingly, 45 samples were correctly genotyped as RhD positive and 17 as RhD negative, in these last

Table 1. The estimated quantity of fetal DNA in maternal plasma.

<table>
<thead>
<tr>
<th>Gestation (weeks)</th>
<th>Fetal DNA (copies number/mL maternal plasma)*</th>
<th>Mean</th>
<th>Range</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>11-14</td>
<td>143</td>
<td>80.88-225.43</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>15-28</td>
<td>371</td>
<td>152.49-755</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>29-40</td>
<td>1363</td>
<td>180.27-3494.66</td>
<td>22</td>
<td></td>
</tr>
</tbody>
</table>

*The number of copies of fetal DNA in maternal plasma was estimated from the mean number of copies of RhD exon 5, 7 and 10.
Fetal RhD genotyping in maternal plasma of RhD- women

Figure 1. Representative real-time PCR amplification plots showing the presence of fetal and maternal DNA in maternal plasma of RhD-negative women pregnant with a RhD-positive fetus (A) and only the presence of maternal DNA in maternal plasma of RhD-negative women pregnant with a RhD-negative fetus (B). Real-time multiplex PCR was performed in duplicate.

cases, no prenatal anti-D prophylaxis is needed and injection of anti-D immunoglobulin can be avoided. However, for the other cases, the pregnant women are subsequently monitored. Most importantly, no false negative result was observed in this study. Thus the sensitivity of the assay was 100% and the specificity was 94.44%.

These high percentage is most likely the result of the use of an efficient protocol for the RhD genotyping: the quantification of fetal DNA was done using a RQ-PCR that incorporate: 1) a pool of plasmid calibrators estimating the PCR efficiency; 2) a β-globin gene serving as a control gene that confirm the presence and quality of DNA in each sample and 3) a positive and negative controls. Thus, the quantitative nature of the test will assures that a maternal signal is not mistaken for a fetal signal. Moreover, this analysis test is efficient since a multiple RhD exons were targeted by RQ-PCR which allows to overcome false positive results and, thus, improve accuracy of fetal RhD genotyping.

The amount of free fetal DNA is obviously a limiting factor for fetal RhD genotyping in maternal blood. It is well documented that the amount of fetal DNA increases during pregnancy [8], as was observed in our study. However, there is a considerable variation in the amount of circulating fetal DNA from pregnancy to pregnancy with the progress of gestational age as indicated in table 1. Our current findings would suggest that accurate RhD genotyping is possible in the first trimester, since the earliest pregnancy in which the presence of fetal DNA was confirmed in our study was 11 weeks. It would be advisable to repeat the test at later stages of gestation to insure fetal genotyping results.

Traditional management for RhD-negative women at risk for RhD alloimmunization involves determining the RhD status of the fetus through chorionic villus sampling from amniotic fluid, and administrating the prophylactic human derived RhD immunoglobulin during pregnancy and after delivery. Both practices carry their own risks and associated costs [16]. Therefore, the feasibility of mass testing for the fetal RhD genotype with maternal plasma is highly desirable for ethical and economical reasons. Indeed, it avoids unnecessary administration of anti-D immunoglobulin, to approximately 40 percent of RhD-negative women bearing RhD-negative fetus [17].

Non-invasive fetal RhD genotyping by targeting multiple exons with real time PCR is highly sensitive and accurate. It’s the best method for assessing RhD fetal status in RhD-negative mothers and represents the first step in identifying fetus at risk for alloimmunization. In conclusion, it will be of interest to plan the practice of this analysis in the routine testing of all RhD-negative pregnant women, this will present surely a significant achievement in the application of research to clinical practice.

Conflicts of interest: none.

References


