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Large-Scale Pre-Diagnosis Study of Fetal *RHD* Genotyping by PCR on Plasma DNA from RhD-Negative Pregnant Women

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Abstract

Background: The routine prenatal determination of fetal RhD blood group would be very useful in the management of pregnancies in RhD-negative women, as up to 40% of these pregnancies bear a RhD-negative fetus. The fetal DNA present in maternal plasma offers an opportunity for risk-free prenatal diagnosis.
Aim: This study focused on the feasibility and accuracy of large-scale RhD fetal diagnosis in non-immunized and anti-D immunized RhD-negative women.
Methods: Plasma DNA was extracted from 893 RhD-negative pregnant women and amplified in exons 7 and 10 of the *RHD* gene using conventional and real-time PCR. The results were then compared with the *RHD* fetal genotype determined on amniotic cells and/or the RhD phenotype of the red blood cells of the infants at birth.
Results: After exclusion of 42 samples from women exhibiting a nonfunctional or rearranged *RHD* gene, fetal RhD status was predicted with a 99.5% accuracy. A strategy is also proposed to avoid the small number of false-positive and -negative results.
Conclusion: Fetal *RHD* genotyping from maternal plasma DNA in different clinical situations may be used with confidence.

The prenatal determination of fetal *RHD* genotype is beneficial in the management of pregnancies in RhD-negative women, whether the women have developed anti-D immunization or not.

In France, as well as in many other countries, repeated screening for anti-D immunization is performed antenatally in the blood of every non-anti-D immunized RhD-negative pregnant women, although 30–40% of the women carry a RhD-negative fetus without the risk of hemolytic disease of the newborn. Furthermore, Rh immunoprophylaxis is systematically provided following invasive prenatal diagnosis procedures such as amniocentesis or chorionic villus sampling, antepartum, and fetomaternal hemorrhages. In some countries, anti-D immunoglobulin administration is recommended during the third trimester of pregnancy even though the RhD blood group of the fetus is unknown.[1] The determination of the fetal *RHD* genotype could modify this lack of rationality in medical practices and prevent unnecessary exposure for the mother to human immunoglobin which is only available in limited amounts. Likewise, it is of prime importance to detect the RhD status of fetuses from anti-D immunized women who may be expecting a RhD-negative fetus when the father is D heterozygote.

The demonstration that the *RHD* gene is absent in the genome of the RhD-negative individuals[2] allowed the prenatal determination of the fetal RhD blood group with PCR amplifications. The first tests described were based on the PCR amplification of a single *RHD* gene region.[3,4] Multiple molecular polymorphisms related to the extreme polymorphism of the RH blood group system, such as DNA segmental exchanges between the *RH* genes for D category variants, were later characterized.[5,6] Recently, a *RHDψ* pseudogene frequently present in RhD-negative Black Africans was also characterized.[7] Therefore, the high level of accuracy and robustness of *RHD* genotyping has been demonstrated when using PCR that amplify several regions of the *RHD* gene.[8-10]

Fetal DNA is generally obtained through invasive means, such as amniocentesis, which carry a risk of spontaneous miscarriage[11] and a significant risk of boosting the maternal immune response to fetal red blood cells antigens.[12,13] Recently the detection of fetal DNA in the plasma and serum of pregnant women[14] has opened up new possibilities for the non-invasive prenatal determination of fetal Rh blood group which are risk-free for the fetus.

Fetal *RHD* genotyping studies using fetal DNA from maternal plasma were initially determined by amplifying a single *RHD* gene region, either with real-time[15-20] or conventional PCR.[21,22] Some recent studies were performed with at least two distinct *RHD*-specific PCR to ascertain the fetal *RHD* genotype in view of the extreme polymorphism of the RH blood group system.[23,24] Less than 140 samples were included in all these reports.

The aim of this study was to validate the diagnostic use of fetal *RHD* genotyping with two PCR amplifications by using fetal cell-free DNA from maternal plasma. Since the major problem with this test is the lack of internal control detecting the presence of fetal DNA when PCR results are negative, we evaluated its true accuracy on a large number of samples from RhD-negative pregnant women treated in different diagnosis situations.

Materials and Methods

Maternal Blood Samples

Non-immunized and anti-D immunized RhD-negative pregnant women were recruited with informed consent either 1–2 weeks before undergoing amniocentesis for karyotyping or during a routine prenatal check-up for antibody titration, respectively.

5–10mL maternal blood samples were collected in tubes containing EDTA. Blood samples were taken from nearly 200 laboratories located all over France, as well as in the West Indies, Tahiti, and Djibouti. The samples were transported by mail or courier and arrived at the laboratory 1–2 days after extraction. Blood samples were centrifuged at 1730g for 10 minutes at room temperature and the plasma was carefully removed, then aliquoted by 800μL into polypropylene cryogenic vials and stored at –20°C until further processing. The buffy coat (50μL) containing maternal leukocytes was removed, washed three times with NaCl 0.9% at 5500g and stored at –20°C until further processing.

RhD Phenotyping on Red Blood Cells from Newborn Blood

The RhD phenotype was determined on blood samples collected at birth for Rh immunoprophylaxis (non-immunized patients) or for the diagnosis of Rh hemolytic disease (anti-D immunized patients). RhD serotyping was performed with commercial anti-RhD reagents (Diagast, Lille, France) using a direct agglutination test.

Plasma DNA Extraction

DNA was extracted from 800μL of plasma sample with a QIAamp® Blood Kit (Qiagen, Courtaboeuf, France) according to the 'blood and body fluid' protocol recommended by the manufacturer. DNA was eluted using 60μL of the elution buffer provided with the kit. For each series of extraction, three controls were added: (i) a positive control from a sample (800μL) of plasma from RhD-positive women diluted (1/500 v/v) with plasma from an RhD-negative man; (ii) a negative control composed of 800μL plasma from an RhD-negative man; and (iii) a blank control for which 800μL of water was used instead of plasma.

Processing of Amniotic Fluid Samples

Amniocentesis was performed several days or weeks after maternal blood sampling, either for karyotyping (non-immunized patients) or the measurement of the bilirubin content of the amniotic fluid (anti-D immunized patients). For *RHD* genotyping, 1mL of amniotic fluid was centrifuged at 5585g for 10 minutes at room temperature and the amniocytes pellet was immediately subjected to DNA extraction.

Genomic DNA Extraction

Fetal or maternal genomic DNA from amniocytes and buffy coat, respectively, were extracted using the InstaGene Matrix (BioRad, Hercules, CA, USA). Briefly, the pellet was resuspended in 200μL of the InstaGene Matrix and incubated for 30 minutes at 56°C followed by 30 minutes in boiling water. After centrifugation at 5585g for 4 minutes at room temperature, the supernatant-containing genomic DNA was removed and stored at 4°C until it was required for further processing.

Conventional PCR Amplification

Two distinct *RHD*-specific PCR (exons 7 and 10) were performed from plasma DNA. Four distinct *RHD*-specific PCR in exons 4, 7, and 10, and intron 4, were performed for *RHD* genotyping from amniocyte and leukocyte DNA.[9] A PCR in *RHD* exon 6 was performed to better characterize a *RHD*ψ allele.[7] All oligonucleotides used are listed in table I.

Each amplification reaction was set up in a final volume of 50μL containing 10μL of plasma or genomic DNA, 1×PCR buffer, 0.25μM of each primer, 200μM each dNTP, 1.5mM $MgCl_2$ (2mM $MgCl_2$ for exon 4 and intron 4 amplification reactions), 1 U Platinum® *Taq* DNA polymerase (Life Technologies, Cergy

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Table I. Characteristics and sequences of oligonucleotide primers used in the *RHD* genotyping

Primers	Nucleotide sequence	Localization	Product size (bp)	Specificity
Genomic DNA				
RHDIN3-F[7]	5′-GCCGACACTCACTGCTCTTAC-3′	Intron 3	208	Dψ
D9-R	5′-CAAACTGGGTATCGTTGCTG-3′	Exon 4	171	D
D6-F	5′-GCCGGGGTGTTGTAACCGAGT-3′	Exon 7	133	D
D7-R	5′-ATTGCCGGCTCCGACGGTATC-3′	Exon 7		
D4-F	5′-GGATTTTAAGCAAAAGCATCCAAGAA-3′	Exon 10	291	D
D5-R	5′-ACTGGATGACCACCATCATATATGC-3′	Exon 10		
P4-F	5′-CGCAGCCTATTTTGGGCTG-3′	Exon 4	111 (IC)	D + CE
P5-R	5′-CCAGCATGGCAGACAAACT-3′	Exon 4		
I2-F	5′-ACGATACCCAGTTTGTCT-3′	Exon 4	1050 (IC)	CE
E5-R	5′-ATCCACAAGAAGAGGGCG-3′	Exon 5	450	D
RH6F[7]	5′-CAAAAACCCATTCTTCCCG-3′	Intron 5	355	Dψ
RHDψ EX6R[7]	5′-AACACCGCACTGTGCTCC-3′	Exon 6		
Plasma DNA				
D6-F	5′-GCCGGGGTGTTGTAACCGAGT-3′	Exon 7	133	D
D7-R	5′-ATTGCCGGCTCCGACGGTATC-3′	Exon 7		
D4-F	5′-GGATTTTAAGCAAAAGCATCCAAGAA-3′	Exon 10	131	D
RDB-R[15]	5′-AGTGCCTGCGCGAACATT-3′	Exon 10		

IC = internal control.

Pontoise, France). 0.5% DMSO was added into the reaction mixture for intron 4 amplification only. Identical thermal profiles were used for all PCR amplifications.

Thermal cycling, performed on a Robocycler® (Ozyme, Montigny le Bretonneux, France) was initiated with 10 minutes of denaturation at 95°C, followed by 40 cycles with denaturation at 92°C for 1 minute 20 seconds, annealing at 57°C for 1 minute 20 seconds and extension at 72°C for 2 minutes, and a final extension at 72°C for 7 minutes. 15μL of PCR products were electrophoresed on a 2% agarose gel-containing ethidium bromide and visualized under ultraviolet light.

Real-Time PCR

Real-time PCR analysis was performed using a LightCycler® apparatus (Roche Molecular Biochemicals, Mannheim, Germany). PCR reactions were performed in the LightCycler® glass capillaries using the LC FastStart DNA Master SYBR® Green I kit (Roche Molecular Biochemicals, Mannheim, Germany) in a final volume of 20μL containing 5μL of plasma DNA, 0.5μM of each primer, 4mM $MgCl_2$, and 2μL of the reaction mix provided with the kit which contained Faststart T*aq* DNA polymerase. Amplification conditions were initial denaturation at 95°C for 8 minutes, followed by 45 cycles with denaturation at 95°C for 15 seconds, annealing at 59°C for 10 seconds, and extension at 72°C for 5 seconds. Melting curves allowing product identification were generated by slowly heating (0.1°C/seconds) from 67–95°C. The *RHD* exon 7-specific and *RHD* exon 10-specific melting temperatures were 86.10°C (SD 0.41°C) and 83.08°C (SD 0.40°C), respectively.

A multiplex *RHD*-specific PCR was also performed on maternal leukocyte DNA (35 cycles of amplification) using the same conditions described above, except the concentrations of the primers (0.15μM for D6-F and D7-R, and 0.5μM for D4-F and RDB-R; see table I).

Results

Improvements of PCR Reactions for the *RHD* Genotyping from Maternal Plasma

Since the RH blood group system is highly polymorphic, we wanted to ascertain the fetal *RHD* genotype with at least 2 *RHD* PCR in distinct regions. Among the 4 PCR we routinely used for fetal *RHD* genotyping from amniocytes (pairs of primers RHDIN3-F/D9-R for exon 4; D6-F/D7-R for exon 7, D4-F/D5-R for exon 10 and I2-F/E5-R for intron 4; see table I), only 2 *RHD* PCR in exons 7 and 10 exhibiting the best sensitivity[9] were tested to detect fetal *RHD* sequences in maternal plasma.

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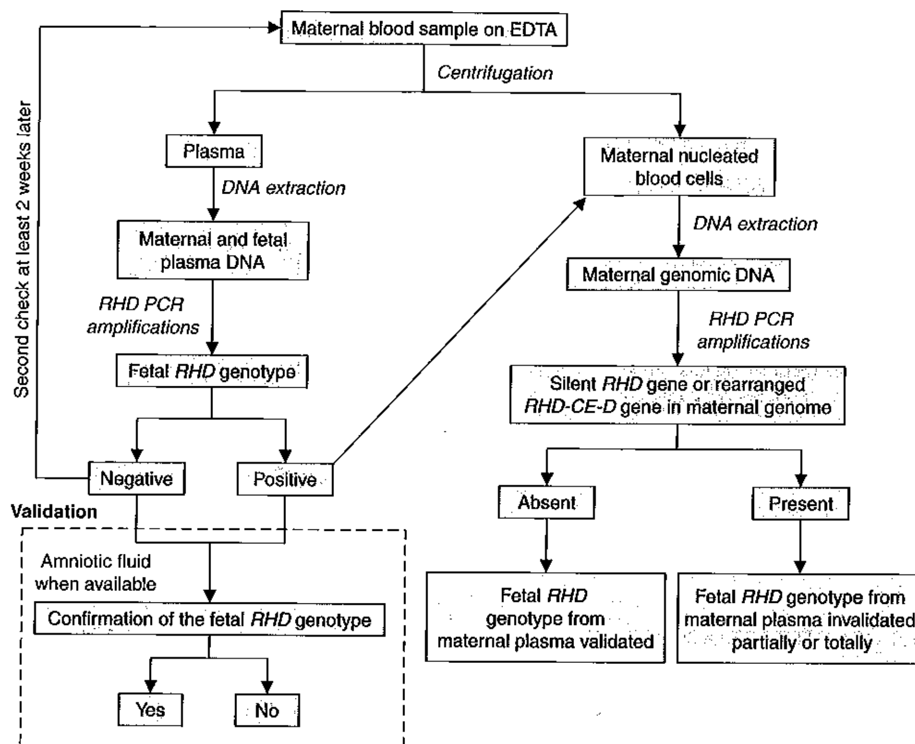

Fig. 1. Chart flow of the maternal blood samples process. Immediately after their receipt, maternal blood samples were centrifuged and plasma samples were aliquoted and stored at −20°C with a buffy coat until needed for further processing. The fetal *RHD* genotype was determined by real-time and conventional PCR. The absence of a silent *RHD* gene or a rearranged *RHD-CE-D* gene in the maternal genome was only checked in those patients whose fetuses exhibited at least a positive result for one *RHD* PCR. Fetal *RHD* genotype from maternal plasma was confirmed by the fetal *RHD* genotype from amniotic cells and/or by the RhD phenotype of the infant at birth.

The results of conventional PCR performed with the first 100 pregnant mothers revealed a significant difference of sensitivity between these two *RHD* PCR reactions. The exon 7 *RHD* PCR was able to detect fetal *RHD* sequences in maternal plasma in pregnancy as early as 7 gestational weeks (gw), whereas the exon 10 *RHD* PCR failed to detect close to 50% of fetal *RHD* sequences from plasma samples tested during the first trimester of pregnancy (data not shown). The sensitivity of the exon 10 *RHD* PCR was, therefore, improved by changing one *RHD* sequence-specific oligonucleotide as outlined by Lo et al.[15] The sensitivity of this new *RHD* exon 10 PCR (with primers D4-F and RDB-R) reached that of the *RHD* exon 7 PCR (data not shown).

We also performed real-time PCR using the LightCycler® apparatus to increase the specificity of the PCR amplifications combined with an extra-level of protection against post-PCR contamination.

Each *RHD* PCR was performed in duplicate, once in conventional PCR and once in real-time PCR. Plasma samples that tested negative or positive in both PCR amplification techniques and in both *RHD* exons were considered RhD-negative or RhD-positive, respectively. When discordant results for one or both PCR between the two amplification techniques were observed, DNA plasma was extracted once more and *RHD* exons 7 and 10 PCR were carried out again with both techniques. *RHD* fetal DNA was considered as present when a positive signal was observed at least for the *RHD* exon 7 PCR, once in both amplification techniques. On the other hand, the fetal *RHD* genotype was undetermined when *RHD* exon 7 PCR was negative and *RHD* exon 10 PCR positive. Indeed, this pattern could correspond either to a RhD-negative but RhC-positive phenotype or to D^{IV} or DBT D category phenotypes that were not identified with these PCR.[25]

RHD Genotyping on Maternal Leukocyte DNA: A Mandatory Step

The concentration of fetal DNA in maternal plasma corresponds to a minor fraction of the total plasma DNA (from 3.4% during the second trimester of pregnancy to 6.2% at late pregnancy).[26] As a consequence, the presence of a nonfunctional *RHD* gene in the maternal genome from phenotypically RhD-negative women invalidates the fetal genotyping from maternal plasma because of the possible false-positive results. Therefore, the presence of a nonfunctional *RHD* gene was checked by a multiplex *RHD* exons 7 and 10 real-time PCR on maternal leukocyte DNA each time a plasma sample tested positive (figure 1). Such silent

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RHD genes in the maternal genome could also be highlighted by the real-time PCR performed on plasma DNA since the exponential growth phase for both PCRs began earlier (approximately 25th cycle) than with the positive plasma DNA control (approximately 35th cycle; figure 2).

Among the 893 women in this study, 34 women (29 anti-D immunized and 5 non-immunized RhD-negative women) were found to carry a nonfunctional *RHD* gene. The fetal *RHD* genotyping on maternal plasma was invalidated for these 34 cases. Three additional conventional PCR in exon 4, intron 4, and exon 6, were performed to better characterize these inactive *RHD* genes. Twenty-six were identified as an African *RHDψ* pseudogene[7] as they exhibited a 208 bp-long fragment in *RHD* exon 4 and T807G nonsense mutation in *RHD* exon 6 (data not shown). The eight remaining inactive *RHD* genes were all found in Caucasian women génomes and their characterization is under investigation.

In addition, 14 RhD-negative but RhC-positive women were positive with the *RHD* exon 10 PCR and negative with the *RHD* exon 7 PCR using genomic DNA (data not shown). These results suggest the presence of a hybrid *RHD-CE(3-7)-D* or *RHD-CE(2-9)-D* allele expressing a dCe[s] or dCe phenotype, respectively. Accordingly, the fetal genotype on maternal plasma was determined as RhD-positive only when a positive signal in *RHD* exon 7 was detected (nine cases) and the *RHD* genotype was undetermined for the five other samples.

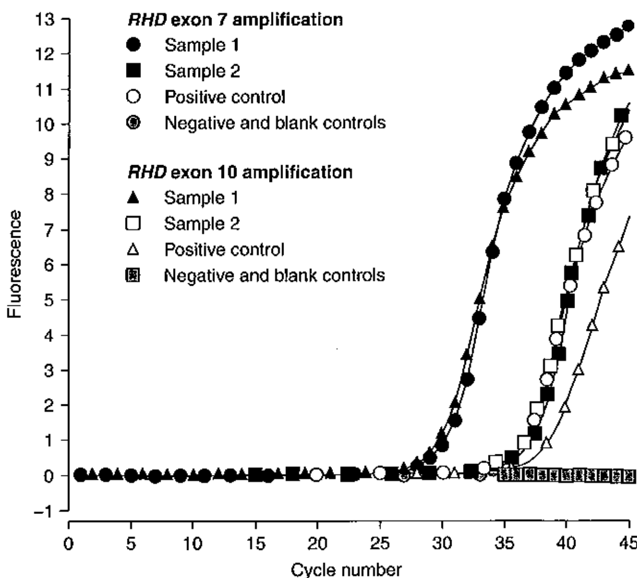

Fig. 2. Detection of the *RHD* exons 7 and 10 with real-time PCR in maternal plasma. Two patients' samples were tested. Sample 1 exhibited a maternal *RHD* silent gene, invalidating the test, and sample 2 gave a positive result. Positive (plasma from RhD-positive women) diluted (1/500 v/v) in plasma from an RhD-negative man, negative (plasma from an RhD-negative man) and blank (water) controls were used.

Table II. Subdivision of maternal plasma samples. For validation, the results of *RHD* genotyping from maternal plasma were compared to *RHD* genotyping of amniotic cells (group I), infant blood at birth (group II) or both (group III)

Validation method	Group I	Group II	Group III
Maternal plasma	+	+	+
Amniotic fluid	+	NA	+
Infant blood	NA	+	+

NA = not available.

Fetal *RHD* Genotyping on Maternal Plasma DNA

Results observed with the earliest plasma sample for each pregnancy were compared with the *RHD* fetal genotype determined on amniotic fluid with four *RHD*-specific PCR (group I) or deduced from the RhD phenotype of the infant at birth (group II) or both (group III) [see table II].

Among the 893 samples of this study, 39 were excluded for the reasons given above and three because an *RHD* exon 10 but no *RHD* exon 7 was detected using plasma DNA.

Therefore, we validated the results of 851 plasma samples: 306 non-immunized women sampled between 7 and 36 gw (see table III); and 545 anti-D immunized women collected between 7 and 40 gw (see table IV).

Six hundred and fifty-four fetuses were predicted to be RhD-positive from PCR on plasma DNA (tables III and IV). *RHD*-positive fetal genotype was confirmed for 649 samples by PCR on amniotic cells and/or red cells phenotype at birth. False-positive results were obtained with 5 samples (table III). The red blood cells of two children (table III) were typed as RhD-negative but these children had inherited a *RHDψ* pseudogene from their father, as indicated by the presence of a 208 bp-long fragment in the *RHD* exon 4 PCR using amniotic cell DNA. Two other children were phenotyped as Rh-negative at birth although *RHD*-positive DNA was found in maternal plasma (table IV). As no amniotic fluid or infant blood was available for *RHD* genotyping, we could not conclude whether these discrepancies could be attributed to the presence of an inactive paternally inherited *RHD* gene. The last discrepancy was observed for a twin pregnancy resulting from assisted medical procreation (table III). RhD-positive DNA was amplified from maternal plasma at 13 gw. Two months later, both fetuses were genotyped as RhD-negative on amniotic cells and the red blood cells of the children were phenotyped as RhD-negative at birth. In this case, a third non-evolutive embryo was suspected to be present at ultrasonography which could have been RhD-positive.

One hundred and ninety-seven fetuses were predicted to be RhD-negative using *RHD* exons 7 and 10 PCR on maternal plasma

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Table III. The accuracy of fetal *RHD* genotyping from maternal plasma of non anti-D immunized pregnant women. Concordant and discordant results refer to fetal *RHD* genotype from maternal plasma which were confirmed or not confirmed, respectively, by *RHD* genotyping from amniotic cells (group I), infant blood at birth (group II), or both (group III)

	Group I				Group II				Group III			
	concordant		discordant		concordant		discordant		concordant		discordant	
	pos	neg	f-pos	f-neg	pos	neg	f-pos	f-neg	pos	neg	f-pos	f-neg
07–11 gw (n = 18)	4	5	0	0	1	2	0	0	4	1	1[a]	0
12–14 gw (n = 88)	33	20	0	1	2	1	0	0	23	7	1	0
15–19.5 gw (n = 155)	66	27	0	0	0	0	0	0	51	10	1[a]	0
20–24 gw (n = 23)	7	5	0	0	0	1	0	0	5	5	0	0
25–28 gw (n = 12)	1	4	0	0	1	2	0	0	4	0	0	0
29–32 gw (n = 4)	0	2	0	0	1	1	0	0	0	0	0	0
>32 gw (n = 6)	1	3	0	0	1	1	0	0	0	0	0	0
Total (n = 306)	**112**	**66**	**0**	**1**	**6**	**8**	**0**	**0**	**87**	**23**	**3**	**0**

a Paternally inherited *RHDψ* allele.

F-neg = false-negative (*RHD*-negative by maternal plasma but *RHD*-positive by other methods; **F-pos** = false-positive (*RHD*-positive by maternal plasma, but *RHD*-negative by other methods); **gw** = gestation weeks; **NA** = not available; **neg** = *RHD*-negative genotype; **pos** = *RHD*-positive genotype.

(tables III and IV). *RHD*-negative fetal genotype was confirmed for 193 samples by PCR on amniotic cells and/or red cells phenotype at birth. False-negative results were obtained with four samples (collected at 7, 7, 12, and 19 gw). They were all identified as RhD-positive when *RHD* fetal DNA was amplified either on a subsequent maternal plasma sample collected 2–4 weeks later or on amniotic cells.

Eight plasma samples (5 mothers exhibiting an *RHD* exon 10 in their genome) were undetermined since *RHD* exon 10 but not exon 7 was amplified. Five of them were found to be RhD-negative when amniotic cells or infant blood were available (data not shown). The remaining three fetuses were genotyped as D^{IV} category on amniotic cells. Serologic and molecular analysis at birth confirmed a D^{IVa} phenotype for the three children (data not shown).

Discussion

This was the largest pre-diagnosis study of fetal *RHD* genotyping using PCR on plasma DNA from Rh-negative pregnant women. Eight hundred and ninety-three cases were analyzed, i.e. the results of *RHD* genotype on amniotic fluid and/or of RhD phenotype on infant blood at birth were available. The result was invalidated in 42 instances: 34 women exhibited a nonfunctional *RHD* gene in their genome and the fetal genotype was further

Table IV. Accuracy of fetal *RHD* genotyping from maternal plasma of anti-D immunized pregnant women. Concordant and discordant results refer to fetal *RHD* genotype from maternal plasma which were confirmed or not confirmed, respectively, by the *RHD* genotyping of amniotic cells (group I), infant blood at birth (group II), or both (group III)

	Group I				Group II				Group III			
	concordant		discordant		concordant		discordant		concordant		discordant	
	pos	neg	f-pos	f-neg	pos	neg	f-pos	f-neg	pos	neg	f-pos	f-neg
07–11 gw (n = 39)	8	0	0	1	10	6	0	0	14	0	0	0
12–14 gw (n = 65)	12	4	0	0	21	2	1	0	24	0	0	1
15–19.5 gw (n = 138)	27	16	0	0	48	12	0	1	29	5	0	0
20–24 gw (n = 107)	17	3	0	0	45	17	1	0	22	2	0	0
25–28 gw (n = 73)	9	0	0	0	42	8	0	0	14	0	0	0
29–32 gw (n = 63)	11	1	0	0	37	7	0	0	7	0	0	0
>32 gw (n = 60)	5	1	0	0	39	12	0	0	3	0	0	0
Total (n = 545)	**89**	**25**	**0**	**1**	**242**	**64**	**2**	**1**	**113**	**7**	**0**	**1**

f-neg = false-negative (*RHD*-negative by maternal plasma but *RHD*-positive by other methods; **F-pos** = false-positive (*RHD*-positive by maternal plasma, but *RHD*-negative by other methods); **gw** = gestation weeks; **NA** = not available; **neg** = *RHD*-negative genotype; **pos** = *RHD*-positive genotype.

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undetermined for eight plasma samples since *RHD* exon 10 but no *RHD* exon 7 were amplified.

As a consequence, we determined fetal *RHD* genotype using both conventional and real-time PCR for 851 samples. The real-time PCR is more convenient for a diagnosis purpose: it is less time-consuming, has an extra level of protection against post-PCR contaminations, and the best specificity of PCR amplification. However, we did not observe real-time PCR to be more sensitive than conventional PCR for the detection of plasma DNA in early pregnancy.

Among the five false-positive results, two were caused by the presence of a paternally inherited RHDψ gene. For two remaining cases the presence of a paternally inherited silent *RHD* gene could not be ascertained since no amniotic fluid nor infant blood was available. Finally, a non-evolutive RhD-positive embryo was suspected to be present for the last case.

The four false-negative results were due to a low fetal DNA concentration since no signal was observed in early pregnancy while *RHD*-positive signals were detected in three plasma samples collected later in pregnancy. These discrepancies highlighted the usefulness of a positive control for the presence of fetal DNA. The search for internal controls has produced some useful options but none appear to be suitable for all samples. Y chromosome-specific PCR, such as SRY, were used by several groups.[22,23,27-29] All these tests exhibited sensitivity close to 100% but were informative only for pregnancies involving male fetuses. The detection of highly polymorphic short tandem repeat (STR) markers is independent of the fetal gender but exhibited a much lower sensitivity than that reported for the fetal *RHD* genotyping from maternal plasma. It reached 70% according to Tang et al.[30] for second trimester pregnancies, whereas Finning et al.[31] have not be able to detect fetal STR alleles in maternal plasma using an 8-locus STR kit. Only Pertl et al.[32] reached a sensitivity of 100% but all 12 pregnancies were more than 37 weeks.

The lack of an internal control to ascertain the fetal *RHD*-negative genotype when no *RHD* PCR signal was detected should be compensated. Firstly, the efficiency of DNA recovery or an inhibitory effect of DNA extract should be checked by introducing low amounts of a plasmid DNA in plasma samples before DNA extraction[24] followed by specific PCR amplification to detect this exogenous DNA in absence of an *RHD* signal. Secondly, as it is known that the concentration of fetal DNA increases with gestational age,[26,33] an initial result of fetal *RHD*-negative genotype should always be confirmed by: (i) repeated fetal *RHD* genotyping tests from a new maternal plasma sample taken two or four weeks later and always beyond the 14th gw; and/or (ii) on amniotic cells if available. In this way, it is noteworthy that we never observed a false-negative result on two consecutive plasma samples from the same pregnant woman.

We performed two distinct *RHD* PCR in exons 7 and 10 to ascertain the fetal *RHD* genotype since the RH blood group system is highly polymorphic. However, the interpretation is uncertain when only PCR *RHD* exon 10 is positive (eight cases in our study). This pattern may suggest the presence of a hybrid *RHD-CE(3-7)-D* or *RHD-CE(2-9)-D* allele found in RhD-negative Black Africans expressing a dCe^s or dCe phenotype, respectively[34,35] (five cases). It could also indicate the presence of a D^{IV} or DBT category alleles,[25] both being the only D category alleles exhibiting no positive signal in *RHD* exon 7 PCR (three cases). In such situation, a third *RHD*-specific PCR in exon 4 should be performed to distinguish these different alleles expressing either an RhD-negative or RhD-positive phenotype. The fetuses would be genotyped as *RHD*-positive or -negative when the *RHD* exon 4 is amplified or not, respectively.

In our study, about 4% (34 out of 893) of RhD-negative women exhibited a silent *RHD* gene in their genome, most (26 out of 34) were carrying a *RHDψ* pseudogene frequently observed in the RhD-negative Black African population.[7] These observations highlight the need to define specific PCR for genotyping the fetuses in such situations. Some groups have already described specific *RHD* PCR that did not amplify the *RHDψ* allele and specific *RHDψ* PCR that did not recognize a normal *RHD* allele.[23,24] Although both assays are applicable to plasma samples, their sensitivity is lower than that of the PCR used for the *RHD* prenatal diagnosis and, therefore, are not currently used in our laboratory.

We also found 0.9% (8 out of 893) of RhD-negative women exhibiting other nonfunctional *RHD* genes, a percentage much higher than that previously found in German blood donors.[36] An independent study consisting in *RHD* genotyping 400 Caucasian RhD-negative women confirmed this ratio, as four women exhibited a silent *RHD* gene that was not a *RHDψ* allele (personal data). Unfortunately, we are still unable to propose any prenatal diagnosis on maternal plasma for these mothers since multiple molecular mechanisms are involved.[36]

Conclusions

Considering our present study, a different diagnostic approach for fetal *RHD* genotyping on maternal plasma would be proposed by distinguishing whether RhD-negative pregnant women were anti-D immunized or not.

Fetal *RHD* genotyping should be proposed for every non anti-D immunized RhD-negative women in order to limit the use of antenatal prophylaxis to only those women bearing an RhD-

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positive fetus. This is possible because the *RHD* fetal genotype can be determined by two *RHD* PCR in exons 7 and 10 in duplicate in real-time PCR. Although some false-positive results might be tolerated in this situation, it is crucial to strictly avoid false-negative results that may result in subsequent immunization of the mother. For example, a fetus exhibiting a *RHD* exon 10 without an *RHD* exon 7 should be considered RhD-positive if antenatal prophylaxis needs to be administer in an emergency. If time allows, a third PCR in the *RHD* exon 4 should be performed to clarify the fetal *RHD* genotype status.

The presence of a paternally inherited *RHDψ* pseudogene in PCR *RHD*-positive fetus represents only a small number of cases and it need not be systematically checked, unless the father is known to be of Black African origins. In the case of negative *RHD* PCR results, confirmation from a second maternal plasma sample should be obtained two weeks later before considering abstention of Rh immunoprophylaxis.

The anti-D prophylaxis is not provide to anti-D immunized RhD-negative women. However, in these situations the diagnosis of RhD incompatibility must be certain and, therefore, the specificity and sensitivity of fetal *RHD* genotyping on maternal plasma should approach 100% because it will guide the degree of intervention in the pregnancy. Every plasma sample that exhibited an *RHD* exon 10 but no *RHD* exon 7 should be further tested in the *RHD* exon 4 to distinguish a dCe^{s} or dCe phenotype from a D^{IV} or DBT D category phenotype, since D^{IVa} fetuses could experience severe hemolytic disease if the mother is anti-D immunized (personal data). Specific *RHDψ* PCR could be performed in order to limit the number of false-positive results for fetuses bearing another *RHD* gene inherited from the father. False-negative results need to be completely avoided and repeated testing is required on at least two plasma samples collected some weeks apart when the PCR result is negative.

In conclusion, we observed a rate of concordance of 99.5% (847 of the 851 samples) between the *RHD* fetal genotype on maternal plasma and that determined by *RHD* PCR using amniotic cells or that deduced from the RhD phenotype on neonatal red blood cells. These results were obtained from a large number of samples and allow us to propose the extension of fetal RhD testing from maternal plasma to a maximum of RhD-negative pregnant patients so that more pregnant women can benefit from the testing.

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