Genotyping approach for non-invasive foetal *RHD* detection in an admixed population

Carolina Trucco Boggione[1,2], Melina E. Luján Brajovich[1,2], Stella M. Mattaloni[1,2], René A. Di Mónaco[3], Silvia E. García Borrás[2], Claudia S. Biondi[2], Carlos M. Cotorruelo[1,2]

[1]Instituto de Inmunología Clínica y Experimental Rosario-Consejo Nacional de Investigaciones Científicas y Técnicas (IDICER-CONICET); [2]Laboratorio de Inmunohematología, Facultad de Ciencias Bioquímicas y Farmacéuticas; [3]Facultad de Medicina, Universidad Nacional de Rosario, Rosario, Argentina

Background. Non-invasive foetal *RHD* genotyping can predict haemolytic disease of the foetus and the newborn in pregnancies with anti-D alloantibodies and also avoid antenatal anti-D prophylaxis in pregnant women carrying an *RHD* negative foetus. Considering that the Argentine genetic background is the result of generations of intermixing between several ethnic groups, we evaluated the diagnostic performance of a non-invasive foetal *RHD* determination strategy to guide targeted antenatal RhD immunoprophylaxis. This algorithm is based on the analysis of four regions of the *RHD* gene in cell-free foetal DNA in maternal plasma and maternal and paternal *RHD* genotyping.

Materials and methods. DNA from 298 serologically D negative pregnant women between 19-28 weeks gestation were *RHD* genotyped. Foetal *RHD* status was determined by real-time PCR in 296 maternal plasma samples. In particular cases, *RHDΨ* and *RHD-CE-D^s* alleles were investigated in paternal DNA. Umbilical cord blood was collected at birth, and serological and molecular studies were performed.

Results. Of the 298 maternal samples, 288 were D–/*RHD*– and 10 D–/*RHD*+ (2 *RHD*DAR*; 5 *RHD-CE-D^s*; 3 *RHDΨ*). Plasma from *RHD*DAR* carriers was not analysed. Real-time PCR showed 210 *RHD*+ and 78 *RHD*– foetuses and 8 inconclusive results. In this latter group, paternal molecular studies were useful to report a *RHD* negative status in 5 foetuses while only 3 remained inconclusive. All the results, except one false positive due to a silent allele *(RHD[581insG])*, agreed with the neonatal typing performed in cord blood.

Discussion. The protocol used for non-invasive prenatal *RHD* genotyping proved to be suitable to determine foetal *RHD* status in our admixed population. The knowledge of the genetic background of the population under study and maternal and paternal molecular analysis can reduce the number of inconclusive results when investigating foetal *RHD* status.

Keywords: prenatal diagnostic, *RHD* genotyping, immunoprophylaxis, admixed population.

Introduction

Haemolytic disease of the foetus and the newborn (HDFN) is a serious and potentially fatal disorder caused by transplacentally acquired maternal antibodies, most frequently directed against the D antigen of the Rh blood group system. The use of antenatal prophylactic anti-D immunoglobulin in all D negative pregnant women and its postnatal administration after delivering a D positive child has greatly decreased the incidence of D alloimmunisation. However, this disorder is still a significant cause of foetal-neonatal morbidity and mortality[1,2]. Non-invasive foetal *RHD* genotyping, based on the analysis of cell-free foetal (cff) DNA in maternal plasma, not only allows an early risk assessment of HDFN in pregnancies with anti-D alloantibodies, but also has the potential to avoid antenatal anti-D prophylaxis in D negative pregnant women carrying an *RHD* negative foetus[3-7].

The accurate prediction of the foetal D type by cff DNA analysis in D negative pregnant women relies on the detection of the prevalent *RHD* variants found in the population under study. The current population of Argentina is the result of generations of intermixing between Native American Indians, Africans and Europeans. We have recently examined the genetic variability of the *RHD* locus and found that more than 2% of D negative individuals harbour an *RHD* allele[8]. We also observed that this proportion reaches up to 5% in certain geographical areas[9]. In all cases, the most frequently encountered variants were *RHDΨ* and *RHD-CE-D^s*, even though *DEL* alleles such as *RHD(46T>C)* and *RHD(M295I)* and different recombined structures,

namely *RHD-CE(3-9)-D*, *RHD(329T>C)-CE(3-9)-D* and *RHCE-RHD($3_{361del11}$-10)*, were also detected. In this scenario, strategies for non-invasive prenatal *RHD* testing must be properly designed to avoid false results. Most of the protocols developed for Europeans involve the amplification of two *RHD* exons and reach sensitivity greater than 99.5%[5,10-15]. Little is known about non-invasive foetal *RHD* genotyping strategies in admixed populations[16]. Considering the genetic background of Argentineans, here we evaluated the diagnostic performance of a non-invasive foetal *RHD* determination strategy to guide targeted antenatal RhD immunoprophylaxis. The proposed protocol is based on the analysis of four different regions of the *RHD* gene in cff DNA and the detection of maternal and paternal *RHD* variants.

Materials and methods

Subjects

Peripheral blood samples were obtained from 298 D negative women with singleton pregnancies. The D negative status of the patients enrolled in this study was assessed by haemagglutination with two commercially available monoclonal anti-D reagents (Rediar, Buenos Aires, Argentina) containing the IgM clone MS-201 and the IgM clone RUM-1, respectively. These anti-Ds show weak reactions with most variant D phenotypes and do not agglutinate DVI red blood cells. All weak reactions were considered negative and the indirect antiglobulin test (IAT) was not performed. The gestational age at the time of blood collection ranged from 19 to 28 weeks. Paternal peripheral blood samples (n=8) were also analysed only when the foetal *RHD* status obtained by real-time PCR was not conclusive. Samples belonged to a cohort of individuals from the city of Rosario, Argentina. Written informed consent was obtained before sample collection in accordance with the Declaration of Helsinki.

Samples

Blood samples (10 mL) were collected into EDTA-containing tubes and centrifuged at 1,500 *g* for 7 minutes within 12 hours after sampling. Plasma aliquots (1.2 mL) were transferred into fresh microtubes without disturbing the buffy coat and centrifuged at 11,000 *g* for 10 minutes. Subsequently, 1.1 mL of supernatants were collected into fresh microtubes and stored at −20 ºC for a period no longer than one month until cell-free DNA extraction.

Maternal buffy coat was used to obtain genomic DNA by a salting out method. In some cases, paternal DNA was also obtained from peripheral blood using the same procedure.

For cell-free DNA isolation, a 1.1 mL plasma aliquot of each sample was thawed at room temperature and centrifuged at 7,000 *g* for 5 minutes to isolate cryoprecipitates. After centrifugation, 1 mL of plasma was processed with the QIAmp Blood Mini Kit (Qiagen, Hiden, Germany), following the "Blood and Body Fluid Protocol" recommended by the manufacturer, increasing the volumes of the reagents proportionately. Adsorbed DNA was eluted with 65 µL of water.

RHD variant analysis in maternal samples

Before foetal *RHD* genotyping, all D negative samples from pregnant women were analysed for the presence of intron 4 and the 3' untranslated region (UTR) of the *RHD* gene using a multiplex PCR strategy, as previously described[17]. In samples containing the two *RHD* specific fragments, the *RHDψ* allele was investigated by PCR[18]. In contrast, in samples containing only the 3' UTR, the *RHD-CE-D^s* allele was examined by a specific PCR that amplifies a *5'RHD-RHCE3'* hybrid exon 3 characteristic of the type 1 variant[19]. If these alleles were not detected, samples were subjected to further molecular analysis to identify the maternal *RHD* variant[20,21].

Foetal *RHD* genotyping

The presence of foetal *RHD* sequences in cff DNA was determined with two duplex and one monoplex real-time PCR assays, using primers and probes previously described (Table I)[22]. *RHD* exon 10 duplexed with *SRY* was performed in triplicate while *RHD* exon 4 duplexed with *RHD* exon 7 and the monoplex assay for *RHD* exon 5 were performed in duplicate. In total, nine *RHD* specific amplifications per sample were performed in each real-time PCR run. Amplification reactions were performed using a Stratagene Mx3000P (Agilent Technologies, Santa Clara, CA, USA). Each PCR (20 µL) consisted of 1x mastermix containing buffer, $MgCl_2$, dNTPs and polymerase (Universal Mastermix, Applied Biosystems, Foster City, CA, USA), 0.3 µM primers, 0.2 µM *RHD* exon 7, 10 and *SRY* probes, 0.05 µM *RHD* exon 4 and 5 probes and 9 µL template. After initial incubation at 50 ºC for 2 minutes, denaturation at 95 ºC for 10 minutes and 45 2-step cycles at 95 ºC for 15 seconds and 60 ºC for 60 seconds were performed[22].

The foetus was predicted to be D positive if at least 7 of the 9 replicates for the *RHD* exons analysed were positive (Ct value <42). Samples not fulfilling this criteria but with positive signals in most *RHD* exons were re-tested. If no replicates or only 1 of the 9 replicates were positive, the foetus was predicted to be D negative. In this case, at least 2 positive replicates for *SRY* were considered necessary to confirm the presence of foetal DNA if the foetus was male. When neither *RHD* nor *SRY* sequences were detected, a foetal *RHD* negative status was reported if the same result was obtained upon re-testing a new sample of maternal

Table I - Primers and probes used for real-time PCR.

	Name	Sequence (5' to 3')
EXON 4	RHDEX4F	CTGCCAAAGCCTCTACACG
	RHDEX4R	ATGGCAGACAAACTGGGTGTC
	RHDEX4 Probe	(FAM)-TTGCTGTCTGATCTTTATCCTCCGTTCCCT-(TAMRA)
EXON 5	RHDEX5F	CGCCCTCTTCTTGTGGATG
	RHDEX5R	GAACACGGCATTCTTCCTTTC
	RHDEX5 Probe	(FAM)-TCTGGCCAAGTTTCAACTCTGCTCTGCT-(TAMRA)
EXON 7	RHD7F*	GGATTCCCCACAGCTCCA
	RHD7R*	CCGGCTCCGACGGTATC
	Exn7 Probe*	VIC-ATGGGCTACAACTTC-MGB
EXON 10	RHD10F*	TGCCTGCATTTGTATGTGAGA
	Exn10R*	AGTGCCTGCGCGAACATT
	RHD10 Probe*	FAM-CATGACAGCAAAGTC-MGB
SRY	SRYF*	GAGCAGCCAGGGAGGCAGAT
	SRYR*	GCAAAACATGGTAATTTAGTAACGTT
	SRY Probe*	VIC-ACTACTTGCCCTGCT-MGB

* Primers and probes sequences for *RHD* exon 7 and 10 and *SRY* genes were kindly provided by Nuria Nogués from the *Banc de Sang i Teixits*, Barcelona, Spain.

plasma collected after three weeks of the first sampling. All other situations were analysed in consideration of the maternal and paternal *RHD* genotype obtained.

RHD variant analysis in paternal samples

In those cases in which the real-time PCR results were not conclusive, the presence of *RHDψ* and *RHD-CE-D^s* was investigated in paternal DNA by previously reported strategies that allow the identification of *RHDψ/RHD*, *RHDψ/RHD deleted, RHD-CE-D^s/RHD* and *RHD-CE-D^s/RHD deleted* genotypes[18,19].

Flow charts for foetal *RHD* genotyping strategy involving maternal, foetal and paternal molecular analyses are summarised in Figures 1 and 2.

Serological and molecular analyses of cord blood samples

Umbilical cord blood samples from neonates were collected at birth and studied by haemagglutination using a commercially available monoclonal blended anti-D reagent (Wiener Lab, Rosario, Argentina) containing IgM clone TH-28 and IgG clone MS-26. When an immediate spin-negative result was observed, the samples were tested by the IAT. The anti-D used reacts with most weak D and partial D red blood cells, including DVI, in the IAT. In some cases, molecular analyses were also performed to determine foetal *RHD* status.

Results

The first step in our foetal *RHD* genotyping protocol was the molecular analysis of maternal DNA. Among 298 pregnant women, 288 D negative/*RHD* negative and 10 D negative/*RHD* positive samples were found. In this latter group, 5 *RHD-CE-D^s*, 3 *RHDΨ* and 2 *RHD*DAR* carriers were identified. *RHD*DAR* carriers were not included in the cohort for prenatal genotyping. Consequently, the protocol was performed in a total of 296 plasma samples.

In the 288 plasma samples from D negative/*RHD* negative pregnant women investigated, 205 *RHD* positive foetuses were identified. In these samples, exons 4, 5, 7 and 10 were positive, with the expected Ct values. In contrast, 76 foetuses were predicted to be *RHD* negative as no *RHD* amplification signals were obtained. Within this latter group, *SRY* positive results (at least 2/3 replicates) confirmed the presence of foetal DNA in 45 cases. In the remaining samples (n=31), amplification of neither *RHD* exons nor *SRY* sequences were obtained. Before reporting a foetal *RHD* negative status, a new plasma sample was re-tested three weeks after the first sampling and the results obtained agreed with the previous analysis. In these cases, the foetal *RHD* status was reported as *RHD* negative and immunoprophylaxis would not be recommended. Cord blood phenotype showed that only 1 sample was falsely predicted antenatally to be D positive. Further molecular characterisation allowed the identification of the previously reported silent *RHD* variant *RHD(581insG)*[8]. In this allele, a G insertion at position 581_582 is responsible for a premature stop codon at position 592-594.

In the rest of the samples (n=7), 4 different situations were observed according to the real-time PCR amplification pattern obtained.

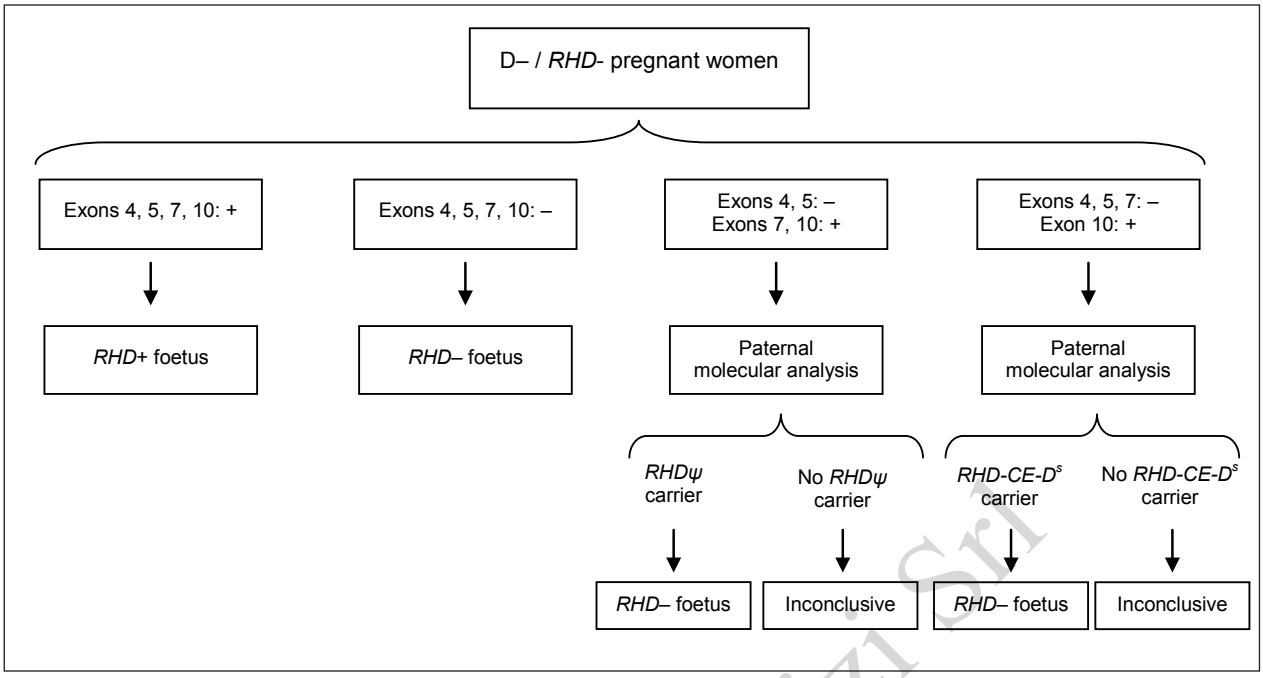

Figure 1 - Flow chart for foetal *RHD* genotyping strategy in D–/*RHD*– pregnant women.

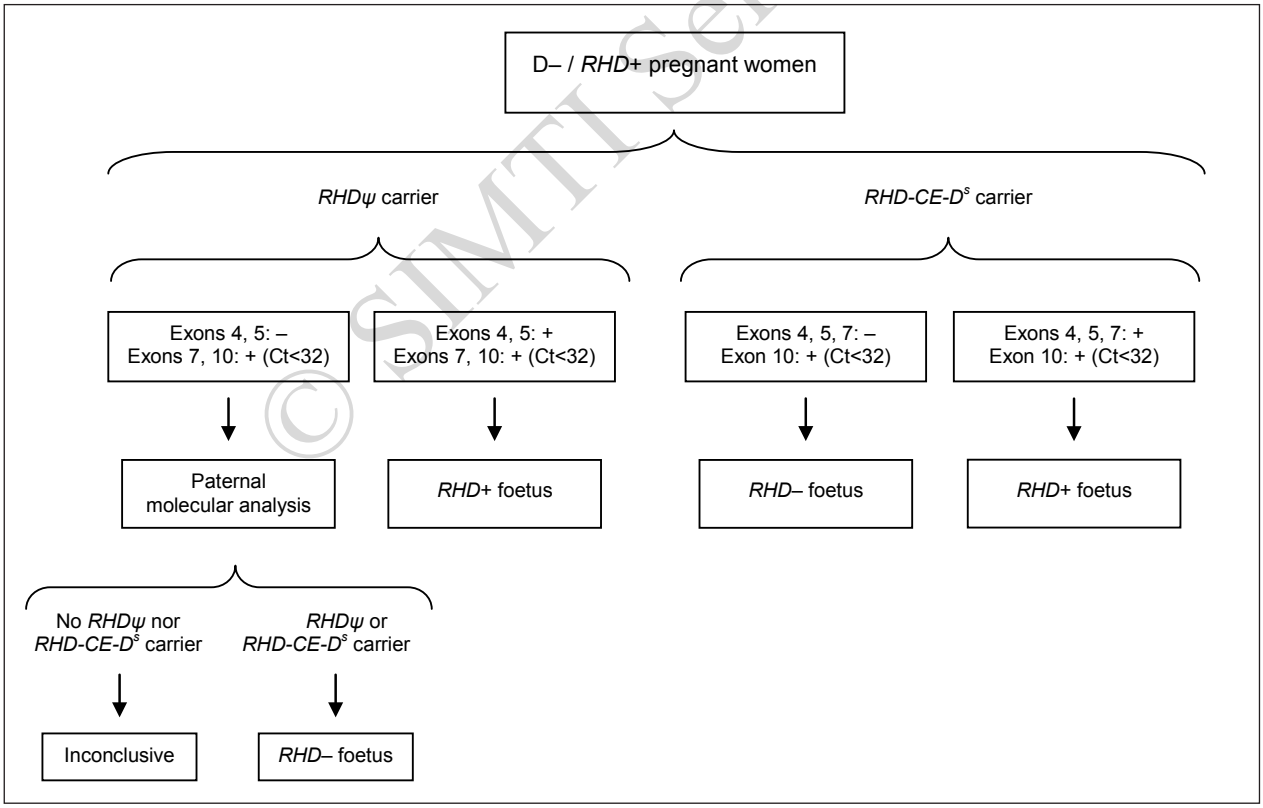

Figure 2 - Flow chart for foetal *RHD* genotyping strategy in D–/*RHD*+ pregnant women.

- Situation 1: in 4 cases, only *RHD* exons 7 and 10 PCR were positive (2/2 and 3/3 replicates, respectively). Paternal analysis revealed that the fathers were D positive and *RHDΨ* carriers (*RHD/RHDΨ* genotype) suggesting that the *RHDΨ* was inherited by the foetuses. Thus, the foetal *RHD* status was reported as *RHD* negative. Serological and molecular analyses of cord blood samples confirmed that the newborns were D negative and *RHDΨ* carriers.
- Situation 2: in one case, only *RHD* exon 7 and 10 PCR were positive (2/2 and 3/3 replicates, respectively). Paternal analysis revealed that the father was D positive and not a *RHDΨ* carrier, suggesting that the foetus could have inherited a different *RHD* allele. Thus, the foetal *RHD* status was reported as *RHD* inconclusive. Serological and molecular analyses of the cord blood sample identified a DVI phenotype in the newborn.
- Situation 3: in one case, only *RHD* exon 10 PCR was positive (3/3 replicates). Paternal analysis revealed that the father was D positive and a *RHD-CE-D^s* carrier (*RHD/RHD-CE-D^s* genotype) suggesting the *RHD-CE-D^s* was inherited by the foetus. Thus, the foetal *RHD* status was reported as *RHD* negative. Serological and molecular analysis of the cord blood sample confirmed that the newborn was D negative and a *RHD-CE-D^s* carrier.
- Situation 4: in one case, only *RHD* exon 10 PCR was positive (3/3 replicates). Paternal analysis revealed that the father was D positive and not an *RHD-CE-D^s* carrier suggesting that the foetus could have inherited a different *RHD* variant. Thus, the foetal *RHD* status was reported as *RHD* inconclusive. Serological and molecular analyses of the cord blood sample showed that the newborn was D negative and a *RHD-CE(3-9)-D* carrier.

Maternal molecular studies revealed the presence of *RHD* alleles in 10 pregnant women. Two of them were *RHD*DAR* carriers and so the prenatal genotyping could not be performed. In the remaining 8 samples, 4 different situations were observed according to the maternal allele found and the real-time PCR amplification pattern obtained.

- Situation A: analysis of maternal DNA revealed the presence of *RHD-CE-D^s* in 2 pregnant women. *RHD* exons 4, 5 and 7 real-time PCR were negative and only *RHD* exon 10 was detected with a high level of amplification (Ct values <32); this signal was attributed to maternal DNA. Thus, the foetal *RHD* status was reported as *RHD* negative in both cases.
- Situation B: maternal molecular analysis revealed that 3 pregnant women harboured an *RHD-CE-D^s* allele. Antenatal genotyping showed Ct values for *RHD* exons 4, 5 and 7 PCR within the expected range for foetal DNA. An abnormally high level of *RHD* exon 10 amplification (Ct values <32) was also observed and attributed to the maternal *RHD-CE-D^s* allele. Thus, the foetal *RHD* status was reported as *RHD* positive in all cases.
- Situation C: molecular analysis in 2 pregnant women revealed the presence of *RHDψ* allele. Antenatal foetal genotyping showed low Ct values (<32) for *RHD* exons 7 and 10, corresponding to maternal DNA and Ct values for *RHD* exons 4 and 5 within the expected range for foetal DNA. The foetal *RHD* status was reported as *RHD* positive in all cases.
- Situation D: in one case, the pregnant woman carried a non-functional *RHDψ*. Antenatal genotyping of the foetus showed no detection of *RHD* exons 4 and 5 and an abnormally high level of amplification of *RHD* exons 7 and 10 (Ct values <32). Paternal analysis revealed that the father was D positive and neither a *RHDΨ* nor an *RHD-CE-D^s* carrier. Thus, the foetal *RHD* status was reported as *RHD* inconclusive since it was not possible to determine whether the foetus had inherited an *RHD* deleted allele or another *RHD* variant responsible for antigen D expression. Serological and molecular analyses of the cord blood sample showed a DVI phenotype in the newborn.

Results obtained by this foetal *RHD* genotyping strategy in 296 pregnant women are summarised in Table II.

Discussion

Here we have evaluated a protocol for non-invasive antenatal *RHD* genotyping with the aim of guiding targeted antenatal RhD immunoprophylaxis in D negative pregnant women from an admixed population. The method used was designed in consideration of our previous work in which we analysed the *RH* locus variability in the study group. In our previous studies, we had observed that a relatively high incidence of D negative individuals carried DEL or silent variants, and that more than 80% of these D negative/*RHD* positive samples harboured *RHDψ* and *RHD-CE-D^s* alleles[8,9]. Therefore, it is important to consider that the presence of these *RHD* variants could lead to false-positive results when foetal genotyping is performed. Taking into account this molecular background, we propose a stringent diagnostic algorithm based on the molecular analysis of maternal *RHD* genotype followed by foetal *RHD* typing by real-time PCR. In some cases, when the results were not conclusive, paternal *RHD* status was also analysed (Figures 1 and 2). This protocol allowed us to report a fully conclusive foetal *RHD* genotype in 293 (99%) of 296 pregnant women. All the samples were tested for the presence of *RHD* exons 4, 5, 7 and 10, as well as for *SRY*. Serological D typing in

Table II - Foetal *RHD* genotyping in plasma samples from 296 D negative pregnant women from an admixed population.

Maternal genotype	*RHD* exons				Paternal genotype	Foetal *RHD* status	N.
	4	*5*	*7*	*10*			
RHD deletion	+	+	+	+	NT	D+	205
RHD deletion	-	-	-	-	NT	D–	76
RHD deletion	-	-	+	+	*RHDψ*	D–	4
RHD deletion	-	-	+	+	No *RHDψ*	I	1
RHD deletion	-	-	-	+	*RHD-CE-D^s*	D–	1
RHD deletion	-	-	-	+	*No RHD-CE-D^s*	I	1
RHD-CE-D^s	-	-	-	+ *(Ct<32)*	NT	D–	2
RHD-CE-D^s	+	+	+	+ *(Ct<32)*	NT	D+	3
RHDψ	+	+	+ *(Ct<32)*	+ *(Ct<32)*	NT	D+	2
RHDψ	-	-	+ *(Ct<32)*	+ *(Ct<32)*	No *RHDψ* No *RHD-CE-D^s*	I	1

Maternal genotype, PCR real-time amplification pattern and paternal genotype are depicted. I: inconclusive; NT: not tested.

cord blood revealed that only one false-positive result was obtained by real-time PCR among the 293 foetal genotype obtained, showing an accuracy of 99.7%.

In the population under study, 2.8% of the individuals are D negative/*RHD* positive[9] while the incidence of D variant phenotypes, which are categorised as D negative in pregnant women, is approximately 0.8%; weak D type 1 and weak D type 4 were the most frequently found phenotypes[21]. Maternal DNA genotyping before performing foetal DNA analysis allowed us to detect 10 D negative/*RHD* positive pregnant women carrying an *RHD* variant that may hinder foetal *RHD* detection. The *DAR* allele was identified in 2 of the samples and so foetal DNA analysis could not be performed since the polymorphisms analysed by real-time PCR are present in this maternal variant compromising foetal detection. The remaining samples (n=8) harboured *RHDψ* and *RHD-CE-D^s* alleles; these data were useful in interpreting the real-time PCR results. The *RHD* variants identified and their frequencies resembled those found in the overall population[9,21].

Among the 296 maternal plasma studied, 8 samples showed an inconclusive real-time PCR amplification pattern (Situations 1, 2, 3, 4 and D). However, in 5 of these cases (Situations 1 and 3), molecular analysis of paternal DNA helped safely predict a *RHD* negative status, proving the importance of studying the paternal genetic background.

The primers and probes used for exons 4 and 5 do not permit amplification of the *RHDΨ*[10] and, therefore, *RHDΨ* carriers will type as exons 4- and 5-negative and exons 7- and 10-positive. However, a *DVI* allele also generates the same pattern of amplification. The detection of *RHDΨ* in paternal DNA, either in hemizygous (*RHDΨ/-*) or heterozygous (*RHD/RHDΨ*) genotypes allowed us to report a foetal *RHD* negative status (Situation 1). When *RHDΨ* was not found in the father (Situation 2), we could not exclude the presence of a foetal *DVI* allele and so reported an inconclusive result.

RHD-CE-D rearranged alleles, such as *RHD-CE-D^s* will type as exons 4-, 5- and 7-negative and exon 10-positive. The detection of *RHD-CE-D^s* in paternal DNA allowed us to report a foetal *RHD* negative status only when exon 10 PCR amplification was observed (Situation 3). When *RHD-CE-D^s* was not found (Situation 4), we inferred that another *RHD* allele could be present in the paternal DNA. Considering the high allelic variability of the *RH* locus in the population under study, the possibility of a novel *RHD* variant cannot be ignored. As this putative allele could be responsible for a variant D antigen expression, the foetal *RHD* status was reported as inconclusive.

In all the cases of D negative pregnant women carrying *RHD* silent alleles, either *RHD* exon 10 or both *RHD* exons 7 and 10 showed an abnormally high level of amplification with Ct values lower than 32 cycles. In *RHD-CE-D^s* carriers, amplification of *RHD* exons 4, 5 and 7 (2/2 replicates for each exon) were considered predictive of an *RHD* positive foetus (Situation B), while in *RHDΨ* carriers, the *RHD* positive status was assigned on the basis of *RHD* exons 4 and 5 positive PCRs (2/2 replicates for each exon) (Situation C).

The absence of amplification of *RHD* exons 4, 5 and 7 in *RHD-CE-D^s* carriers allowed us to report an *RHD* negative foetus (Situation A) whereas the absence of amplification of *RHD* exons 4 and 5 in *RHDΨ* carriers

do not allow us to report an *RHD* negative status since the foetus could have inherited a *DVI* allele from the father (Situation D).

The protocol used for non-invasive prenatal *RHD* genotyping was proved suitable for determining foetal *RHD* status in an admixed population within a gestational range of 19-28 weeks. In most of the cases, a conclusive foetal *RHD* status was obtained even when foetuses (Situations 1 and 3) or pregnant women (Situations A, B and C) are silent *RHD* variant carriers (*RHDΨ* or *RHD-CE-D*^{s}). Based on the high sensitivity and specificity of the protocol, if the algorithm applied (Figures 1 and 2) leads to a foetal *RHD* negative result, the antenatal administration of immunoprophylaxis would not be recommended. On the other hand, if the foetal status results *RHD* positive or inconclusive (foetuses carrying DVI or other variants; Situations 2, 4 and D), antenatal anti-D immunoprophylaxis would certainly be recommended. We are aware that, in cases in which real-time PCR shows a negative result for *RHD* and *SRY*, the lack of cff DNA control may lead to the risk of a false negative result caused by its low level in maternal plasma. We agree with other authors[6,23,24] that in pregnant women of these gestational ages, the *RHD* negative status can be confirmed on a second plasma sample collected a few weeks later. However, more stringent criteria are needed when testing D-immunised pregnant D negative women. In these situations, the incorporation of a cff DNA control, such as the *RASSF1A* promoter region sequence, would be recommended to confirm the presence of foetal DNA[4,25,26].

Conclusions

In conclusion, if this strategy is applied with stringent criteria and controls, a specificity of 98.8% and a sensitivity of 100% is achieved. Knowledge of the genetic background of the population under study, and maternal and paternal molecular analysis can reduce the number of inconclusive foetal *RHD* status results. Studies are ongoing to assess the costs and benefits of a routine non-invasive prenatal *RHD* genotyping in all D negative pregnant women in our population to target antenatal RhD immunoprophylaxis.

Funding and resources

This work was supported in part by a grant from the *Agencia Nacional de Promoción Científica y Tecnológica* (Project PICT 2010-1070), Argentina. Funding was also received from the *Universidad Nacional de Rosario*, Argentina.

Authorship contributions

CMC conceived and designed the study, analysed and interpreted the data, and drafted the manuscript. RADM collected the samples. CTB carried out the molecular genetic studies, analysed and interpreted the data, and drafted the manuscript. MELB and SMM helped with DNA isolation and molecular genotyping. SEGB and CSB carried out the immuno-haematologic assays.

The Authors declare no conflicts of interest.

References

1) Moise K, Argoti P. Management and prevention of red cell alloimmunization in pregnancy: a systematic review. Obstet Gynecol 2012; **120**: 1132-9.
2) de Haas M, Thurik F, Koelewijn J, et al. Haemolytic disease of the fetus and newborn. Vox Sang 2015; **109**: 99-113.
3) Daniels G, Finning K, Martin P, et al. Fetal blood group genotyping: present and future. Ann NY Acad Sci 2006; **1075**: 88-95.
4) Hyland CA, Gardener GJ, Davies H, et al. Evaluation of non-invasive prenatal *RHD* genotyping of the fetus. Med J Aust 2009; **191**: 21-5.
5) Scheffer P, van der Schoot C, Page-Christiaens G, et al. Noninvasive fetal blood group genotyping of rhesus D, c, E and of K in alloimmunised pregnant women: evaluation of a 7-year clinical experiencie. BJOG 2011; **118**: 1340-8.
6) Clausen F, Damkjaer M, Dziegiel M. Noninvasive fetal RhD genotyping. Transfus Apher Sci 2014; **50**: 154-62.
7) Chitty LS, Finning K, Wade A, et al. Diagnostic accuracy of routine antenatal determination of fetal *RHD* status across gestation: population based cohort study. BMJ 2014; **349**: g5243.
8) Trucco Boggione C, Luján Brajovich M, Tarragó M, et al. Molecular structures identified in serologically D– samples of an admixed population. Transfusion 2014; **54**: 2456-62.
9) Luján Brajovich M, Trucco Boggione C, Nogués N, et al. [RHD null alleles in RhD negative patients from a hospital of Rosario.] Rev Arg Transf 2010; **36**: 125-30. [In Spanish.]
10) Finning K, Martin P, Soothill P, et al. Prediction of fetal D status from maternal plasma: introduction of a new noninvasive fetal *RHD* genotyping service. Transfusion 2002; **42**: 1079-85.
11) Rouillac-Le sciellour C, Piullandre P, Gillot R, et al. Large-scale pre-diagnosis study of fetal *RHD* genotyping by PCR on plasma DNA from RhD-negative pregnant women. Mol Diagn 2004; **8**: 23-31.
12) Clausen F, Krog G, Rieneck K, et al. Reliable test for prenatal prediction of fetal RhD type using maternal plasma from RhD negative women. Prenat Diagn 2005; **25**: 1040-4.
13) Hromadnikova I, Vechetova L, Vesela K, et al. Non-invasive fetal *RHD* exon 7 and exon 10 genotyping using real-time PCR testing of fetal DNA in maternal plasma. Fetal Diagn Ther 2005; **20**: 275-80
14) Müller SP, Bartels I, Stein W, et al. The determination of the fetal D status from maternal plasma for decision making on Rh prophylaxis is feasible. Transfusion 2008; **48**: 2292-301.
15) Minon J, Gerard C, Sentere J, et al. Routine fetal *RHD* genotyping with maternal plasma: a four year experience in Belgium. Transfusion 2008; **48**: 373-81.
16) Amaral D, Credidio D, Pellegrino J Jr, et al. Fetal *RHD* genotyping by analysis of maternal plasma in a mixed population. J Clin Lab Anal 2011; **25**: 100-4.
17) Cotorruelo C, Biondi C, Borrás SG, et al. Molecular determination of RhD phenotype by DNA typing: clinical applications. Ann Clin Biochem 2000; **37**: 781-9.
18) Singleton B, Green C, Avent N, et al. The presence of an *RHD* pseudogene containing a 37 bp duplication and a nonsense mutation is present in most Africans with the RhD negative blood group phenotype. Blood 2000; **95**: 12-8.

19) Daniels GL, Faas BH, Green CA, et al. The VS and V blood group polymorphisms in Africans: a serologic and molecular analysis. Transfusion 1998; **38**: 951-8.
20) Gassner C, Schmarda A, Kilga-Nogler S, et al. *RHD/CE* typing by polymerase chain reaction using sequence-specific primers. Transfusion 1997; **37**: 1020-6.
21) Luján Brajovich M, Trucco Boggione C, Tarragó M, et al. Comprehensive analysis of *RHD* alleles in Argentineans with variant D phenotypes. Transfusion 2012; **52**: 389-96.
22) Finning K, Martin P, Daniels G. The use of maternal plasma for prenatal RhD blood group genotyping. Methods Mol Biol 2009; **496**: 143-57.
23) Rouillac-Le sciellour C, Sérazin V, Brossard Y, et al. Noninvasive fetal *RHD* genotyping from maternal plasma. Use of a new developed Free DNA Fetal Kit RhD®. Transfus Clin Biol 2007; **14**: 572-7.
24) de Haas M, van der Schoot. Prenatal Screening. ISBT Science Series 2013; **8**: 6-10.
25) Zejskova L, Jancuskova T, Kotlabova K, et al. Feasibility of fetal-derived hypermethylated *RASSF1A* sequence quantification in maternal plasma--next step toward reliable non-invasive prenatal diagnostics. Exp Mol Pathol 2010; **89**: 241-7.
26) White H, Dent C, Hall V, et al. Evaluation of a novel assay for detection of the fetal marker *RASSF1A*: facilitating improved diagnostic reliability of noninvasive prenatal diagnosis. PLoS One 2012; **7**: e45073.

Arrived: 26 August 2015 - Revision accepted: 9 December 2015
Correspondence: Carolina Trucco Boggione
Laboratorio de Inmunohematología
Facultad de Ciencias Bioquímicas y Farmacéuticas
Universidad Nacional de Rosario
Suipacha 531
2000 Rosario, Argentina
e-mail: ctrucco@fbioyf.unr.edu.ar