Original article

Algorithm development and diagnostic accuracy testing for non-invasive foetal *RHD* genotyping: an Indian experience

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Background - The discovery of the cell-free foetal DNA (cffDNA) circulating in the maternal plasma enabled prediction of foetal RHD thus eliminating the risks associated with invasive procedures. Non-invasive foetal *RHD* genotyping has now become the standard approach in developed countries for management of alloimmunised women and is also used for targeted antenatal prophylaxis in non-alloimmunised women.

Materials and methods - cffDNA was extracted from the plasma of 217 RhD negative pregnant women at a gestational age of 10-34 weeks. The foetal *RHD* genotype was determined by real-time polymerase chain reaction (real-time PCR) amplification of exons 4, 5 and 10 in duplicates. After an initial 54 samples, foetal typing was carried out with *RHD* exons 5 and 10 for the remaining samples. *CCR5*, *SRY* and *RASSF1A* genes were used as controls. Results were compared with cord blood serological typing at birth.

Results - Out of the 217 women, 193 were non-immunised and 24 were alloimmunised. A conclusive diagnosis was obtained in 203 samples. Diagnosis was inconclusive in 14 samples; of these, foetal *RHD* genotype could be resolved in six samples after maternal and paternal *RHD* genotyping. A 100% diagnostic accuracy, sensitivity and specificity were demonstrated in 209 women who had had a conclusive result. When the inconclusive samples were included, diagnostic accuracy and sensitivity were more than 95% and specificity was 78.95%.

Discussion - Anti-D is still the leading cause of haemolytic disease of the foetus and the newborn in India. There is, therefore, a need to establish and develop an algorithm for antenatal RhD negative women in India. The positive results of non-invasive foetal *RHD* genotyping, from the start of the 10th week of gestation using two *RHD* exons giving 100% diagnostic accuracy, show promise for routine diagnostic use to the benefit of the antenatal RhD negative Indian population.

Keywords: *cell-free foetal DNA (cffDNA), haemolytic disease of the foetus and the newborn (HDFN), prenatal diagnosis, RHD genotyping.*

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INTRODUCTION

Haemolytic disease of the fetus and newborn (HDFN) has been a major obstetric problem worldwide leading to foetal morbidity and mortality[1]. The advent of postnatal prophylaxis

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in 1968 reduced the rate of alloimmunisation from 13-16% to approximately 0.5-1.8%[2]. A further reduction of 0.14-0.2% was observed in western countries after the combination of antenatal and postnatal prophylaxis[3]. Unfortunately, morbidity due to HDFN is still prevalent in other parts of the world, due to a lack of systematic prenatal care and awareness of this disease.

However, ethical concerns regarding the human origin of RhIg and its use in women carrying a D-negative foetus have been raised[4]. The traditional methods of assessing the foetal blood group although useful, involved risk to the foetus[5]. Hence, foetal *RHD* genotyping by non-invasive means using cell-free foetal DNA (cffDNA) obtained from maternal plasma has found increasing acceptance, with developed countries starting to use it as a diagnostic procedure[6-9].

One of the major mechanisms responsible for RhD negativity is *RHD* gene deletion. cffDNA typing exploits this fact to predict foetal RhD status. The frequency of D-negativity and the genetics responsible for this, such as *RHD* deletion, *RHD* pseudogene (*RHDΨ*), hybrid gene *RHD-CE-D*^{s}, etc., differ because of geographic and ethnic factors[10]. These are the driving forces behind the initiative to design a strategy for foetal *RHD* genotyping. In the Indian population, the frequency of D negativity is 5-7%. It has been shown that in the D^{-}, C/E^{+} Indian population, *RHD* deletion was found to be 69% with *RHD-RHCE-RHD* hybrids, frameshift, nonsense and missense mutations making up the rest[11]. A comprehensive picture involving the type of D-negative alleles in ce positive samples is still awaited. In India, anti-D is the most common antibody found in alloimmunised women. Our institute is a reference centre for immunohaematology in India. We, therefore, identified the need to develop an algorithm and establish a facility for non-invasive foetal *RHD* genotyping to benefit Indian antenatal RhD negative women.

MATERIALS AND METHODS

Subjects

This study was carried out between 2015 and 2018 at the Department of Transfusion Medicine of the ICMR-National Institute of Immunohaematology (NIIH), Mumbai, India, after ethical approval of the institutional Ethics Committee. RhD negative antenatal women referred to the centre for routine indirect antiglobulin test (IAT) for antibody screening followed by Rh antibody titration if required, along with their husband's blood, were recruited for the study after detailed clinical obstetric history and informed consent. Blood samples (10 mL in ethylene diamine tetraacetic acid [EDTA] and 5 mL in a plain vacutainer) from gestational week 8 to 40 were collected from RhD negative women. Only RhD negative women with a RhD positive partner were recruited for the study. Samples from partners of the selected women (5 mL in EDTA and 3 mL in plain vacutainer) were collected. The previous blood grouping report was referred to in cases in which the husband's sample was not available.

Sample preparation and serology

The EDTA blood sample underwent an initial spin at 1,600 × *g* for 10 min. The maternal plasma was then separated and subjected to a further spin at 16,600 × *g* for 10 minutes. 1.1 mL aliquots were stored at −20°C until further processing. Samples received after a maximum of 5 days transportation were rejected. Maternal genomic DNA was extracted from the buffy coat by the phenol-chloroform method[12]. Red cells were used for Rh grouping and Rh phenotyping, and serum for routine IAT for antibody screening by manual gelcard (ID card-LISS/Coombs, Bio-Rad Laboratories, Inc., Cressier, Switzerland) using a commercial three-cell antibody screening panel (ID-Diacell I-II-III, Bio-Rad Laboratories, Inc.). Antibody identification was carried out by manual gelcard (ID card-LISS/Coombs, Bio-Rad Laboratories, Inc.) using ID-Diapanel (Diamed, Cressiur FR, Switzerland). Rh antibody titration was performed by tube technique. Two different commercial monoclonal anti-Ds (anti-D RHESOLVE RUM-1 [IgM] and RHOFINAL anti-D Rho [IgM+IgG], Tulip Diagnostics, Goa, India) were used for Rh typing by direct spin and IAT according to the manufacturer's instructions. In case of a discrepant RhD result, the samples were further tested by a panel of 12 monoclonal anti-D IgG reagents from ALBAclone advanced partial RhD typing kit (Alba Bioscience, Eysins, Switzerland). Rh phenotyping was performed using anti-C, anti-c, anti-E and anti-e antisera (MS24, MS33, MS260, MS16, MS21 and MS63; Bio-Rad, Diamed GmbH). Blood grouping and Rh phenotyping were also carried out on the husband's sample, and RhD zygosity was determined by the most probable genotype and quantitative multiplex polymerase chain reaction (PCR) of short fluorescent fragments (QMPSF)[13].

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Foetal DNA extraction and real-time PCR

The 1.1 mL aliquots were thawed at room temperature and subjected to a spin at 16,600 × *g* for 10 min. Foetal DNA was extracted from 1 mL of the spun plasma using a Qiagen Minelute virus spin kit (Qiagen, Valencia, CA, USA) and was eluted in a final volume of 55 uL[14]. Foetal *RHD* status was determined by real-time PCR because of its high sensitivity on the ABI StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using Takyon™ ROX Probe 2X MasterMix dTTP blue (Eurogentec, Seraing, Belgium) and Taqman hydrolysis MGB probes (Applied Biosystems) (*Online Supplementary Content*, **Table SI**). *RHD* exons 4, 5 and 10 were selected for the assay with the primer and probe sequence of exons 4 and 5 such that it would not permit the amplification of the *RHDΨ* gene. Sex determining Region Y (*SRY*) was used as a foetal DNA marker/control for male pregnancies. *RHD* exon 4/10 and *RHD* exon 5/*SRY* were run as duplex. Chemokine receptor 5 (*CCR5*) was used as a total DNA extraction control. It is also an indicator for the presence of RhD variants and silent maternal *RHD* alleles based on the Ct difference between *CCR5* and *RHD* exon 10. In case of a female Rh negative foetus, Ras association domain family 1 isoform A (*RASSF1A*) was used as a foetal DNA control to ascertain the presence of the foetal DNA. All reactions were run in duplicates except for the gene *CCR5*. Genomic DNA of an RhD positive male at concentration of 0.02 ng/uL and RhD negative female at 1 ng/uL concentration were used as a positive and negative control respectively. No template control (NTC) was run. Control and test samples were run concurrently for optimal assay performance. Strict anti-contamination measures were implemented.

The paternal genomic DNA was extracted using QIAamp DNA Blood Mini Kit (QIAgen GmbH, Hilden, Germany). Supplemental tests, such as Indian specific *RHD* genotyping PCR, QMPSF, and Sanger sequencing were to be used for resolving inconclusive results if required[13,15,16].

Results' interpretation criteria

Results were interpreted on the basis of the cycle threshold (Ct) values and the number of replicates (*Online Supplementary Content*, **Table SII**). Ct values >42 were considered negative. Each target Ct value falling between 34-41 was considered positive. Anything <34 was rechecked and compared with the CCR5 Ct values (<2 Ct difference) to look for maternal variant gene, if any. For a particular target, a sample was scored positive or negative when both the replicates were respectively positive or negative. When a single replicate was found to be positive/negative for a particular target it was repeated. After about a quarter of the sample size, foetal *RHD* genotyping was carried out excluding exon 4. Foetal *RHD* genotyping results were compared with cord blood serology reports, which is the gold standard reference test.

RESULTS

A total of 244 RhD negative antenatal women were registered for the study after blood grouping and IAT for antibody screening. Eight were excluded: three had a miscarriage, two underwent an abortion, and the rest were lost to follow-up (**Figure 1**).

Out of the remaining 236 samples, samples from 8-9 weeks and after 34 weeks were excluded from an evaluation of antenatal prophylaxis and diagnostics, and only 217 samples falling between the start of gestational week 10 to end of week 34 were considered (**Figure 1**). **Table I** shows the characteristics of the 217 women.

Out of the 217 maternal samples tested, foetal RhD status was conclusive in 203 samples and inconclusive in 14 (6.45%). Foetal *RHD* genotyping performed in 203 maternal samples was concordant with the cord blood status, predicting 175 (86.21%) RhD positive and 28 (13.79%) RhD negative offspring (**Figure 2**). There were 110 male and 93 female offspring. Of the 203 maternal samples showing conclusive results, 179 were non-immunised and 24 alloimmunised (**Figure 2**). In the alloimmunised group, the Rh zygosity status of the husband was available for 14 women of which five were found to be hemizygous by

Table I - *Characteristics of 217 RhD negative antenatal women*

Characteristic	Number
Age group, years (median)	19-42 (32.5)
Repeat pregnancy	3
Nulliparous	135
Multiparous	82
Twin pregnancy	2
Non-immunised	193
Alloimmunised	24
Ce positive	28 (12.09%)
cE positive	4 (1.84%)
ce positive	185 (85.25%)

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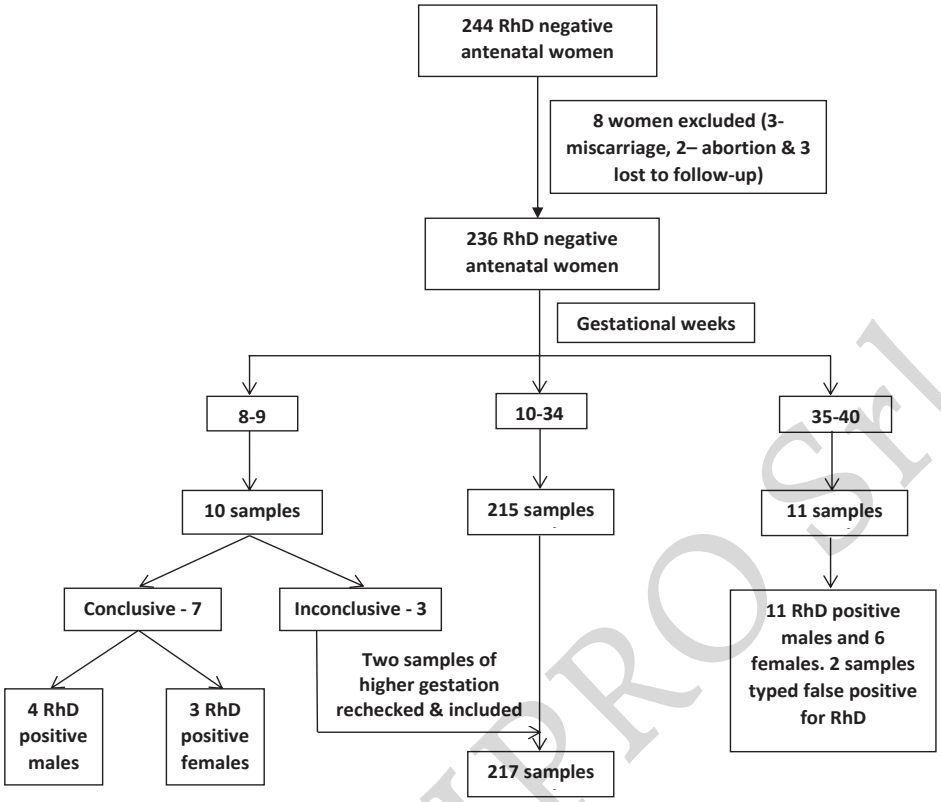


Figure 1 - Sample selection based on gestational age

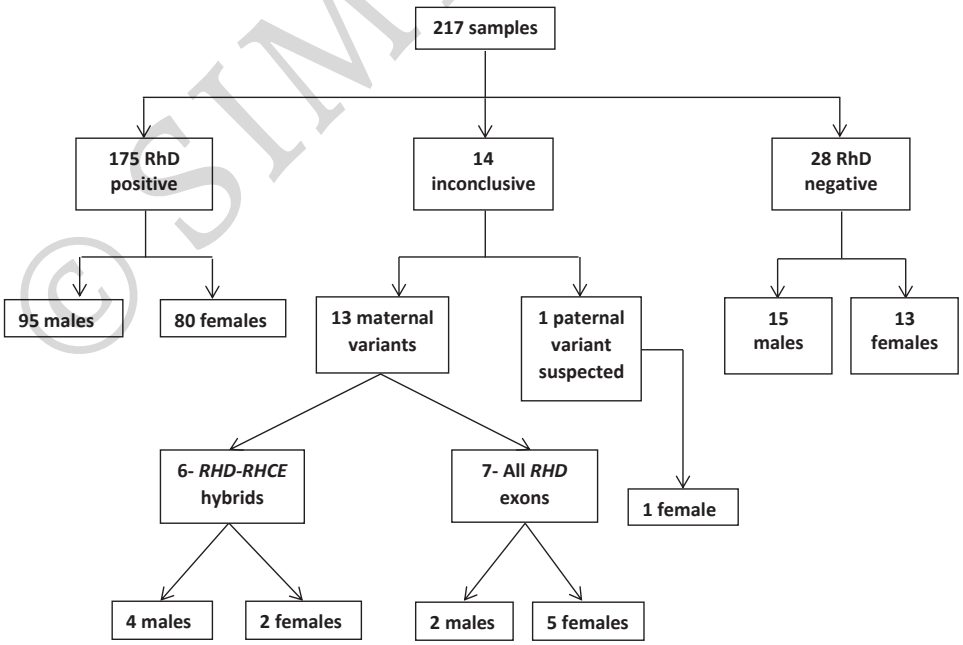


Figure 2 - Results for foetal *RHD* genotyping in 217 samples

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both methods. The antibody was identified as anti-D. All the alloimmunised women (*Online Supplementary Content*, **Table SIII**) gave birth to D-positive neonates which had been correctly predicted.

Statistical analysis

None of the conclusive reports showed false positive or false negative results. Sensitivity and specificity of the assay were both 100% (95% CI: 100-100%) for the *RHD* gene in this study. The positive predictive value (PPV) and the negative predictive value (NPV) were 100% (95% CI: 100-100%). Overall, diagnostic accuracy of the assay was 100%. For the *SRY* gene, 100% (95% CI: 100-100%) sensitivity and specificity were obtained. Diagnostic accuracy including the inconclusive samples was 93.55% (95% CI: 90.21-96.89%).

Indeterminate/inconclusive samples

Indeterminate or inconclusive results were obtained in 14 samples (6.45%) (**Table II**). Samples showing any of the below-mentioned results were re-tested on a second aliquot and, if possible, on a second sample. 1) Positive Ct values for a single exon; 2) considerable Ct difference (≥2) between any two exons; 3) low Ct values for all the exons (≤33); 4) Ct difference of <2 between CCR5 and exon 10. In such cases, foetal RhD status was not predicted as, in the presence of a maternal RhD variant or an RhD^{-}/*RHD*$^{+}$ silent allele, positive amplification curve for *RHD* exons cannot be attributed to the foetal DNA alone. This was confirmed by performing supplemental tests such as the Indian-specific *RHD* genotyping assay, QMPSF, and Sanger sequencing on maternal and paternal genomic DNA.

Table II - *Cycle threshold (Ct) values of samples showing inconclusive results*

Sample n.	*RHD* ex 4 Ct	*RHD* ex 5 Ct	*RHD* ex 10 Ct	Cord blood *RHD* status
1	NT	Negative	<33	Negative
2	Negative	Negative	<33	Negative
3	NT	35	<33	Positive
4	NT	35	<33	Positive
5	NT	36	<33	Positive
6	NT	36	<33	Positive
7	NT	<33	<33	Positive
8	NT	<33	<33	Positive
9	<33	<33	<33	Positive
10	NT	<33	<33	Positive
11	<33	<33	<33	Positive
12	NT	<33	<33	Positive
13	NT	<33	<33	Positive
14	NT	Negative	36	Negative

NT: not tested.

Based on the amplification/no amplification of *RHD* exon 5 (which was missing in all the maternal hybrid alleles) in samples 1-6, the foetal *RHD* genotype could be predicted as the paternal *RHD* alleles also were found to be wild-type (**Table III**). This reduced the number of inconclusive samples to eight (3.69%). Diagnostic accuracy including the inconclusive samples after applying the supplemental tests increased from 93.55% (95% CI: 90.21-96.89%) to 96.31% (95% CI: 93.75-98.87%). Sensitivity and specificity were 95.72% (92.76-98.68%) and 78.95% (65.73-92.17%), respectively.

In cases 7-13 (sample n. 7-13), a maternal *RHD* allele was suspected owing to low Ct values for all the exons and <2 Ct difference between *CCR5* and *RHD* exon 10. In these cases, maternal genomic DNA was subjected to QMPSF which showed the presence of *RHD* exons 1-10. In case 11 (sample n. 11), QMPSF showed the presence of exon 3 duplication (weak D type 150). *RHD* exon sequencing is being pursued for the other samples. Sample n. 14 showed positive Ct values for exon 10 alone. Maternal genomic DNA analysis revealed *RHD* gene deletion. Inheritance of a paternal *RHD* hybrid allele was suspected. Unfortunately, paternal DNA was not available for further analysis.

DISCUSSION

Around 20 years ago, prenatal diagnosis received a major impetus with the demonstration of male foetal DNA sequences in maternal plasma[6]. However, although non-invasive prenatal testing (NIPT) has been used for diagnostic purpose in developed countries, it has not been used in clinical practice in India to guide management of RhD negative pregnancies. In this study, real-time PCR-based genotyping strategy has been developed and standardised for non-invasive foetal *RHD* genotyping in pregnant RhD negative Indian women. High sensitivities ranging from 99% to 99.9% for gestational weeks 10-11 and 25 weeks respectively have been demonstrated in large-scale studies. Over time, greater detection sensitivity and diagnostic accuracy has been observed at a lower gestational age compared to the earlier studies[17-19].

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Table III - *Revised foetal RHD genotyping results for inconclusive samples*

Sample n.	*RHD* ex 4 Ct	*RHD* ex 5 Ct	*RHD* ex 10 Ct	Maternal *RHD* allele	Maternal Rh phenotype	Paternal *RHD* allele	Foetal *RHD* genotyping	Cord blood status
1	NT	Negative	<33	*RHD-CE-(3-9)-D*	Ccee	WT	**Negative**	Negative
2	Negative	Negative	<33	*RHD-CE-(4-9)-D*	Ccee	WT	**Negative**	Negative
3	NT	35	<33	*RHD-CE-(4-9)-D*	Ccee	WT	**Positive**	Positive
4	NT	35	<33	*RHD-CE-(3-8)-D*	Ccee	WT	**Positive**	Positive
5	NT	36	<33	*RHD-CE-(3-8)-D*	Ccee	WT	**Positive**	Positive
6	NT	36	<33	*RHD-CE-(3-8)-D*	Ccee	WT	**Positive**	Positive
7	NT	<33	<33	No exonic mutation present	Ccee	WT	Inconclusive	Positive
8	NT	<33	<33	No exonic mutation present	ccee	WT	Inconclusive	Positive
9	<33	<33	<33	All *RHD* exons present	ccee	WT	Inconclusive	Positive
10	NT	<33	<33	All *RHD* exons present	Ccee	SU	Inconclusive	Positive
11	<33	<33	<33	Weak D type 150	Ccee	WT	Inconclusive	Positive
12	NT	<33	<33	All *RHD* exons present	Ccee	SU	Inconclusive	Positive
13	NT	<33	<33	All *RHD* exons present	ccee	WT	Inconclusive	Positive
14	NT	Negative	36	*RHD* negative (gene deletion)	ccee	Suspected SU	Inconclusive	Negative

Ct: cycle threshold: NT: not tested; WT: wild-type; SU: sample unavailable.

False negatives

In this study, seven samples out of ten belonging to 8 weeks and 9 weeks gestational age gave concordant results for RhD status. Lack of interpretable results (2/4 and 1/4 positive results for *RHD* and 1/2 for *SRY* gene) and late Ct values were seen in three samples termed inconclusive. The uninterpretable results could be due to the paucity of foetal DNA. van der Schoot *et al.* reviewed samples from different studies belonging to weeks 11-13 and found a sensitivity of 99.12%[8]. In an online survey organised by the Worldwide Initiative for Rh Disease Eradication (WiRhE), in which thirteen European countries participated, it was observed that foetal RHD genotyping was not initiated before week 10 of gestation[20]. It is a known fact that foetal DNA concentration is directly proportional to gestational age and increases as the pregnancy progresses. However, although this is true, inter-individual variations in foetal DNA concentrations have been reported depending on the other clinical conditions present[9].

False positives

In previous studies, false positives have mainly been attributed to the presence of maternal RhD^{-}/*RHD*$^{+}$, weak or non-expressing *RHD* alleles in the foetal DNA, cord blood contaminated by maternal blood, vanishing twin, mistyping or mislabelling of cord blood, external DNA contamination[21,22]. In our study, two samples belonging to weeks 35 and 36 gave false positive results. Foetal sex was correctly interpreted. On being typed as false positive, to ascertain our results, we decided to repeat the assay with a second aliquot. Similar results were obtained. Unfortunately, further contact could not be established to corroborate our readings with a blood sample from the neonate.

Assay design

The *RHD* gene is one of the most polymorphic and complex of the blood group genes. The phenotypic variants (apart from D positive, i.e., D negative, weak, partial and DEL) arise from different molecular mechanisms such as deletions, duplications, *RHD-CE-D* hybrid formation, mutations like missense, nonsense, frameshift, etc. In Caucasians, *RHD* gene deletion is the major mechanism for D-negativity. Thus, assay design involving a single exon should prove successful in most cases. However, if used in other populations, it could also lead to mistyping. For example, only exon 7 or exon 10 would type positive for *RHDΨ*, whereas only exon 4 or 5 assay would type

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negative for DFR and DVI variant in some cases. In African, Brazilian, Tunisian, and other mixed ethnicities such as Argentinian, incorporation of two exons in the assay design is essential in order to avoid false positives and false negatives as RhD$^-$/*RHD*$^+$ (*RHD* gene positive but phenotype negative) alleles apart from *RHD* deletion are responsible for D negativity. In view of this, Boggione *et al.* studied the type of *RHD* alleles present in the Argentinian population and then designed an assay for an admixed population incorporating two exons plus an additional two for the indeterminate[23]. With the use of multiple exons, a positive/negative result is reaffirmed. However, the disadvantage of this is the increase in the number of inconclusive results. Nevertheless, this situation is much better than eliciting a false positive or a false negative as samples reported inconclusive are recommended antenatal RhIg prophylaxis[8,9]. Whether the design involves single or multiple exons depends on the type and frequency of RhD variants and silent *RHD* alleles in a given population and should, therefore, be population driven[8,9].

A limitation of this study was that we did not know the molecular heterogeneity of the *RHD* alleles (both RhD$^-$/*RHD*$^+$ and RhD variants) in our population at the time of the assay design. Therefore, initially we adopted a cautious approach and worked with three *RHD* exons[14]. In this way, *RHDΨ* would not be amplified as the primer design does not permit the amplification of exons 4 and 5, but exon 10 is amplified. A similar result would also be seen in case of an *RHD-CE-D* hybrid involving exons 4 and 5. Such cases would be reported as inconclusive. Concordant results obtained with the initial 54 samples (except two), encouraged us further to work with only two regions: exons 5 and 10.

Foetal *RHD* genotyping using exons 5 and 10 has proven successful in our population. The assay has yielded promising results, with 100% diagnostic accuracy, sensitivity and specificity for 209 samples (samples on which a conclusive report could be given) from 10-34 weeks gestational age. The 6.45% inconclusive samples, which can be considered a limitation of the study, give food for thought. The basic premise for NIPT is the fact that foetal *RHD* gene detection is facilitated by the absence of the maternal *RHD* gene. Hence, the number of inconclusive results will be lower in Caucasians, as *RHD* gene deletion is the major mechanism of RhD negativity. In populations with mixed ethnicity (Spanish, Argentinian, etc.), the number of inconclusive results increases, as presence of *RHDΨ*, hybrids and other point mutations (RhD$^-$/*RHD*$^+$ alleles) also contribute to RhD negativity (**Table IV**)[19,23-29].

Table IV – *Comparison of inconclusive results with other studies*

Population[ref]	Sample size	Weeks gestation	*RHD* exons	N. inconclusive	Maternal variant	Foetal (paternal) variant	Insufficient n. of replicates
French (Caucasian)[19]	416	10-14	10	9 (2.2%)	9 (2.2%) [6 African or Caribbean origin]	-	-
Finnish (Caucasian)[24]	10,814	24-26	5 and 7	86 (0.8%)	60 (0.6%) [RHD Ψ-18, DAU- 4]	13 (0.1%)	13 (0.1%)
British (Caucasian)[25]	1,869	8-38	5 and 7	64 (3.42%)	25 (1.34%) [10(*RHD* Ψ), 1 (*RHDDVI*), 1*RHD* (722C>T, Thr241Ile)]	8 (0.43%)	31 (1.66%)
Italian (Caucasian)[26]	284	24-28	5, 7 and 10	9 (3.17%)	4 (1.41%) [*RHD**01W.29, *RHD**11, *RHD*CE(3-7)-D, RHD-CE(2-9)-D*]	-	5 (1.76%)
Brazilian (Mixed)[27]	55	12-29	5 and 7	5 (9.09%)	5 (9.09%)	-	-
Brazilian (Mixed)[28]	185	8-28	5 and 7	7 (3.8%)	7 (3.8%) [5 (*RHD* Ψ),2(*DAU5*)]	-	
Spanish (Mixed)[29]	284	24	5 and 7 6 and 10†	7 (2.5%) [3 Caucasian and 4 Latin American]	7 (2.5%) [3 (*RHDDVI type 1 or 4*), *4 (RHD-CE-D*)]	-	-
Argentinean (mixed)[23]	296	19-28	4, 5, 7 and 10	8 (2.7)	8 (2.7%) [5(*RHD-CE-D*‡); 3 (*RHDΨ)*] Reduced to 3 (1.01%)§	-	-
Indian (present study)	217	10-34	4‡, 5 and 10	14 (6.45%)	13 (5.99%) [6 (*RHD-CE-D*), 1 (Weak D type 150), 6 (in process)] Reduced to 7 (3.23%)§	1 (0.46%)	-

N/n: number; †some samples; ‡54 samples only; § resolved after supplemental tests (maternal and paternal genotyping).

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The molecular mechanisms responsible for D negativity in Indians were not elucidated till 2018. Our group has recently shown that 69% of D^-, C/E^+ Indians have two copies of a deleted *RHD* gene. In this group, 31% showed the presence of an *RHD* gene; 28.7% of these 31% showed the presence of an *RHD-CE-D* hybrid and the rest were single nucleotide variations. The study only discusses D^-C/E^+ variants but it points to the important fact that, apart from D deletion, there are a number of other mechanisms of D negativity in the Indian population[11]. Thus, in our study, the presence of RhD^-/RHD^+ alleles (hybrids), point mutations, etc., in the maternal DNA mainly contributed to the inconclusive results, and these results are comparable to those from other non-Caucasian and mixed populations.

In our study, 14 (6.45%) samples were found to be inconclusive. Thirteen samples harboured a maternal *RHD* allele. On performing the supplemental tests, six maternal samples were found to carry the *RHD-CE-D* hybrid allele. It was shown that foetal RhD status can be predicted in such cases[30]. Thus, the number of inconclusive results was reduced to eight (3.69%). Ten (76.92%) out of the 13 samples (maternal *RHD* gene positive) were positive for the 'C' antigen. Six out of the 10 samples were *RHD-CE-D* hybrids. This means that among the total 28 'C' positive samples in the sample size of 217, 10 (35.71%) 'C' positive samples carried a maternal allele. This is comparable to our earlier study showing presence of an *RHD* allele in 31% of RhD negative C/E^+ samples[11].

Both single[19,31,32] and two *RHD* exon assays have given good results, with over 96% sensitivity and specificity[8]. However, an extensive literature review on studies showing 100% sensitivity (sample size above 200) showed that all studies but one used two or more exons[19,23,24,29,33-38]. In our study, the use of more than one exon enabled us to pick up *RHD* hybrids. In addition, use of exon 10 alone could have led to three false positives being typed out of the 14 inconclusive samples according to cord blood serology. Thus, having the foresight to use more than one exon proved correct and informative.

CONCLUSIONS

Anti-D still remains a leading cause of HDFN in India the major reasons for which are a lack of uniformity in antenatal prophylaxis administration throughout the country and inadequate antenatal care. The chance that an RhD negative woman would carry an RhD negative foetus is around 26% in Indian RhD negative women; when the partner is hemizygous RhD positive, figures are around 21%. Thus, from the clinician's point of view, 21% non-immunised RhD negative women could be spared RhIg prophylaxis. In case of RhD alloimmunised women with high antibody titres, the pregnancy needs to be monitored weekly by ultrasound, specifically assessing the foetal middle cerebral artery peak systolic velocities (MCA-PSV). In such pregnancies, early intervention by foetal *RHD* genotyping can bypass the use of invasive techniques such as CVS and amniocentesis, and help in early management of pregnancy. Should the foetus be genotyped as RhD negative, such women are spared intensive pregnancy management. In case of an inconclusive result, antenatal RhIg prophylaxis is indicated, since the chance of carrying an RhD positive foetus is 50% and prophylaxis ensures the protection of the current and future pregnancies. The risk of social and ethical issues of RhIg administration in case the foetus is RhD negative is far less than the risk of sensitisation of the RhD negative pregnant woman in case of missed antenatal RhIg prophylaxis when carrying an RhD positive foetus.

Our institute is a reference centre in India for immunohaematology, and so antibody screening, Rh antibody titration, and weak D testing are part of the routine workup. Hence, the safety afforded by non-invasive foetal *RHD* genotyping, and also the high sensitivity and diagnostic accuracy associated with it, led us to establish this facility in India to benefit Indian RhD negative non-immunised and alloimmunised women. We demonstrated its feasibility and clinical utility in the Indian population using two regions of the *RHD* gene from the start of week 10 of gestation which would enable early diagnosis. This is particularly important from the point of view of the reproductive health of alloimmunised women. We have also successfully shown that foetal *RHD* status prediction is possible in the presence of a hybrid *RHD-RHCE-RHD* maternal gene.

Modification to strategy

Genomic DNA of those positive for C and E (after phenotyping) should be subjected to QMPSF of the *RHD* gene before carrying out real-time PCR. Three possibilities emerge on genotyping of

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these samples: 1) complete *RHD* gene deletion; 2) presence of all *RHD* exons; and 3) deletion of some *RHD* exons or *RHD-RHCE* hybrid. In this study, it has been shown that, apart from complete *RHD* gene deletion, foetal *RHD* status can also be successfully predicted in case of deletion of some *RHD* exons or *RHD-RHCE* hybrid, thus saving time. All maternal DNA samples should be subjected to QMPSF if time and resources permit. Thus, a simple QMPSF assay could reduce the number of inconclusive samples, avoid unnecessary prophylaxis in a D-negative foetus, and also reduce test turnaround time.

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AUTHORSHIP CONTRIBUTIONS

DP and SK designed the study. DP performed the experiments. DP and SK analysed the data. DP wrote the manuscript. SK and MM revised the manuscript critically. All Authors have approved the final manuscript.

The Authors declare no conflicts of interest.

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