Frequency and characterization of *RHD* variants in serologically D– Surinamese pregnant women and D– newborns

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BACKGROUND: Numerous *RHD* variant genes affect the expression of D on the red blood cell surface. In Suriname, 4.3% of pregnant women were D–, ranging from virtually zero to 7% among ethnic groups. Characterization of *RHD* variants, which are associated with a variable potential to induce anti-D, is of practical clinical importance especially in case of limited access to preventive measures. Here we report on the occurrence of *RHD* variant genes in Surinamese serologically D– pregnant women and their D– newborns from different ethnic groups.

STUDY DESIGN AND METHODS: The RheSuN study is a cross-sectional cohort study in D– pregnant women and their newborns, who visited hospitals in Paramaribo, Suriname, during routine pregnancy care. The presence of *RHD* variants was investigated using quantitative polymerase chain reaction targeting *RHD* Exons 5 and 7 and *RH*-multiplex ligation–dependent probe amplification.

RESULTS: Seven *RHD* variant genes were detected in 35 of 84 women and four *RHD* variant genes in 15 of 36 newborns. The *RHD*03 N.01* and *RHD*08 N.01* variants represented 87% of a total of 62 variant genes. Variants were comparably frequent among ethnicities. In four cases genotyping would have changed anti-D prophylaxis policy: one woman with a *RHD*01EL.01* variant, not associated with anti-D formation and three D– newborns with *RHD*09.01* and *RHD*09.03.01* variants, potentially capable of inducing anti-D.

CONCLUSION: *RHD* variants at risk for anti-D are common among serologic D– individuals from African descent in Suriname. While genotyping D– women has limited added value, it may be considered in newborns from D– women.

D antibodies can lead to severe hemolytic transfusion reactions and hemolytic disease of the fetus and newborn. To prevent anti-D formation, D– red blood cells are transfused in D– recipients and anti-D prophylaxis (RhIG) is administrated to D– women pregnant of a D+ child.

The D antigen is highly polymorphic and numerous *RHD* variants have been described.[1,2] Next to the normal D+ and D– expression, three types of variant D expression exist: weak D, Del, and partial D. Weak D is characterized by low expression of the D antigen (<5000 antigens compared to >10,000 normal expression). The *DEL* type express even lower D antigen levels (<50 antigens), only detectable by absorption-elution techniques. Individuals with partial D express D antigens that lack one or several of the 30 D-epitopes, which result in variable serologic reactivity with D antisera.

ABBREVIATIONS: qPCR = quantitative polymerase chain reaction; *RH*-MLPA = *RH*-multiplex ligation–dependent probe amplification.

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The serologic D– status varies from 15% in Caucasians to 8% in Africans to less than 0.5% in Asians.[3] The molecular mechanisms producing the D– phenotypes differ among races. While in almost all Caucasians the D– status is caused by homozygous deletion of the *RHD* gene, this only accounts for 19% of D negativity in Africans, where a pseudogene *RHD* (*RHD*08 N.01*) and a hybrid *RHD*DIIIa-CEVS(4-7)-D* gene (*RHD*03 N.01*), both not producing D antigens, are present in 66 and 15% of D– individuals, respectively.[4] In 10% to 33% of Asians the *DEL* type is found in serologic D– individuals.[5,6]

To determine the D-status of blood donors, transfusion recipients and pregnant women, serologic typing is still the standard method to prevent D immunization. Individuals with *RHD* variants expressing low or no D-antigens are at risk for D alloimmunization after exposure to D antigens by transfusion or pregnancy. Vice versa, *RHD* variants can also elicit an anti-D response in D– individuals.[7,8]

Characterization of *RHD* variants, in particular those with the potential to induce anti-D, is of practical clinical importance. Genotyping is increasingly applied to predict the expression of D antigens and the risk for D alloimmunization in patients and pregnant women.

The population of Suriname is a mixture of different ethnic groups. Maroons (descendants from mainly Central and West Africans that escaped slavery and established independent societies; the present-day population has the highest level of genetic African origin of Suriname ethnicities[9]), Creoles (mixed-race descendants from African and European ancestry), Hindustani (descendants from contract workers originating from the North-West part of India), and Javanese (descendants from contract workers originating from the Central and the Island of Java, Indonesia) represent the four most common inhabitants. In Suriname, the D status of pregnant women and newborns is only serologically determined using a single anti-D and postpartum anti-D prophylaxis for women at risk for D antibodies is not routine. Our previous study revealed that 4.3% of pregnant women in multiethnic Suriname were serologic D–, ranging from virtually zero to 7% among ethnic groups.[10] Anti-D was present in 12% of multigravida D– women.[11] To explore the need for RhIG to D– pregnant women, we investigated the frequency and characterization of *RHD* variants in a multiethnic cohort of serologically D– women and D– newborns in Suriname.

MATERIALS AND METHODS

Study design

The RheSuN study is a cross-sectional cohort study in D– pregnant women and their newborns, who visited one of the four hospitals (Academic Hospital Paramaribo, Diakonessen Hospital, 's Lands Hospital, and Sint Vincentius Hospital) in Paramaribo Suriname during routine pregnancy care. Inclusion was performed between April 2015 and June 2016.

In Surinamese hospitals, pregnant women and newborns (in umbilical cord or neonatal blood) were routinely tested once for the D antigen using tube direct agglutination tests with monoclonal antiserum (Clones MS26; TH28; Sanquin) or ABO/D+ reverse grouping ID-cards (anti-D cell lines LHM 59/20 (LDM3) + 175-2) or with ABO/D for newborn ID-cards (anti-D cell lines ESD-1M, 175-2; Diaclon, Bio-Rad).

D– women were asked to participate in the RheSuN study. From participants, maternal and/or umbilical or neonatal blood was separated into plasma and remainder cells (DNA) and shipped to Sanquin, the Netherlands, for *RHD* genotyping. For all newborns, written informed consent was obtained from their mothers. The Commission for Human Research of Suriname's Ministry of Health approved this study (VG-022-14). This study was conducted according to the principles of the Declaration of Helsinki.

RHD genotyping

Genomic DNA was extracted from the cells using a using DNA extraction kit (QIAamp Blood Mini Kit, Qiagen Benelux B.V.) according to the manufacturer's instructions. A *RHD* multiplex Taqman quantitative polymerase chain reaction (qPCR), targeting *RHD* Exons 5 and 7, and a qPCR targeting the single-copy gene *albumin*, as previously described was performed, with the modification that a dual-labeled probe 5'-VIC-CTGGCCAAGTTTCAA-3'-NFQ-MGB for *RHD* Exon 5 and 5'-FAM-AGCTCCATCATGGGCTA-3'-NFQ-MGB for *RHD* Exon 7 was used.[12] Both PCR procedures were performed on a PCR system (StepOnePlus, Applied Biosystems) in a total volume of 25 μL containing 12.5 μL Fast Advanced Master Mix 2× (ThermoFisher Scientific), 300 nmol/L of each primers, 100 nmol/L of each probe, and 25 ng of DNA. PCR conditions were as follows: 2 minutes at 50°C and 10 minutes at 95°C for initial DNA denaturation and polymerase activation, followed by 50 cycles of 15 seconds at 95°C and 1 minute at 63°C (for *RHD* Exon 5 and Exon 7) or 1 minute at 60°C (for *albumin*).

In samples with qPCR Exon 5 and/or 7 Ct values below 40 a variant *RHD* was suspected and further analyzed with the *RH*-multiplex ligation–dependent probe amplification (*RH*-MLPA) assay to determine the molecular basis, as previously described.[12] Samples with both exons absent (Ct ≥ 40) were first tested with *RH*-MLPA in pools of five to six DNA samples, and when a pool result was positive for a variant, samples were genotyped individually. DNA from inconclusive samples (i.e., serologically D– and *RH*-MLPA *RHD*01*) were sequenced to test for variant sequences across the 10 *RHD* exons and flanking intron regions not included in the *RH*-MLPA assay. Sequence products were analyzed on a genetic analyzer (3730, Applied Biosystems).

Statistical analysis

Categorical variables are presented as numbers and percentages with 95% confidence intervals (95% CIs).

RESULTS

Between April 2015 and June 2016, a total of 283 pregnant D– women were asked for participation to evaluate anti-D immunization. From these, blood samples were obtained from 84 women and 192 newborns, of which in 40 cases from both. Of the newborns 154 were D+ (80%) and 38 D– (20%). From the latter, 36 samples were available for *RHD* genotyping. From the total of 120 D– samples, 11 were from mother–newborn pairs.

RHD variant genes

Homozygous *RHD* gene deletion was present in 46 out of 84 serologic D– women (55%) and three women had the *RHD*01/ RHD*01 N.01* genotype. In 35 women (41%; 95% CI, 31%-53%) seven different *RHD* variants were detected, for a total of 45 variant genes (Table 1). The *RHD*03 N.01* and *RHD*08 N.01* variants represented 87% of variant genes. From the 10 women with two variants, two were homozygous *RHD*08 N.01*, two were homozygous *RHD*03 N.01*, one was compound heterozygous *RHD*09.02/RHD*01 N.20,* and five women had, next to *RHD*03 N.01* alleles, *RHD*08 N.01* (n = 2), *RHD*01EL.01, RHD*09.01,* and *RHD*10.00* alleles (one each).

In the 36 newborns, homozygous *RHD* gene deletion was present in 17 (47%) and four had the *RHD*01/RHD*01 N.01* genotype. In 15 newborns (42%; 95% CI, 26%-59%) four different variants were detected, for a total of 17 variant genes (Table 1). The *RHD*03 N.01* and *RHD*08 N.01* variants represented 82% of variant genes. One child was homozygous *RHD*08 N.01*, one was compound heterozygous for *RHD*09.01/ RHD*03 N.01*, and 13 children had the variant next to *RHD*01 N.01*. Sequencing of the seven samples with an apparently wild-type *RHD* allele by *RH*-MLPA revealed no mutations that explained D– serology.

Self-declared ethnicity was known for 115 individuals. Although numbers were small, no *RHD* variants were found in the eight Hindustani and the six individuals from other or unknown ethnicities. Among Maroons, Creoles, and mixed ethnicity, variant genes were comparably frequent. Homozygous or compound heterozygous variants, predominantly the *RHD*-null, were present in three (20%) individuals of mixed ethnicity, three (9%) creoles, and six (10%) maroons (Table 2, Table S1, and Fig. S1, available as supporting information in the online version of this paper).

From the 11 mother–newborn sample pairs (seven maroons and four creoles), four mothers and six newborns had 13 variant genes (four *RHD*03 N.01* and nine *RHD*08 N.01*). One child had the variant inherited from the mother, three children from their fathers, and one from mother and father, and in one child the variant could have been inherited from the mother or father (Table S2, available as supporting information in the online version of this paper).

DISCUSSION

In this first study on *RHD* variants, in serologically D– pregnant women and D– newborns in multiethnic Suriname, combined

TABLE 1. *RHD* alleles in serologically D– pregnant Surinamese women and D– newborns

RHD allele (ISBT notation)	Allele name	Number (%) of alleles* Women (n = 84)	Newborns (n = 36)
*RHD*01 N.01*	*RHD* deletion	120 (71)	51 (71)
*RHD*01*	Wild-type *RHD*	3 (1.8)	4 (5.6)
*RHD*03 N.01*	*RHD*DIIIa-CEVS (4-7)-D*	21 (13)	7 (9.7)
*RHD*08 N.01*	*RHD*Pseudo-gene or RHD*Ψ*	19 (11)	7 (9.7)
*RHD*09.03.01*	Weak D type 4.0	0	2 (2.8)
*RHD*09.01*	DAR(T203A)	1 (0.6)	1 (1.4)
*RHD*09.02*	DAR-E	1 (0.6)	0
*RHD*01 N.20*	RHD(G314 V)	1 (0.6)	0
*RHD*10.00*	RHD*DAU0	1 (0.6)	0
*RHD*01EL.01*	RHD(G1227A)	1 (0.6)	0

* Denominator for calculating the percentage of *RHD* alleles was 168 for women and 72 for newborns; All women, except those with *RHD*01* and *RHD*01EL.01* alleles were at risk for anti-D formation; in the newborns *RHD*01*, *RHD*09.03.01* and *RHD*09.01* are potentially capable of inducing anti-D.

results showed that 53% had homozygous *RHD* gene deletions, 6% had wild-type *RHD,* and eight different variant genes were detected in 41%. The *RHD*03 N.01* and *RHD*08 N.01* variants represented 87% of variant genes. Homozygous or compound heterozygous variants were present in 10% of our cohort. While the majority of mother with variants are at risk for anti-D and variants in the children would not induce anti-D, in four cases genotyping would have changed anti-D prophylaxis policy: one woman with a *RHD*01EL.01* variant, not at risk for anti-D formation and three newborns with *RHD*09.01* and *RHD*09.03.01* variants, potentially capable of inducing anti-D in their mothers.

The population of Suriname is a mixture of different ethnic groups. Maroons (descendants from mainly Central and West Africans who escaped slavery and established independent societies; the present-day population has the highest level of genetic African origin of Suriname ethnicities[9]), Creoles (mixed-race descendants from African and European ancestry), Hindustani (descendants from contract workers originating from the North-West part of India), and Javanese (descendants from contract workers originating from the Central and the Island of Java, Indonesia) represent the four most common inhabitants. We detected *RHD* variants exclusively and comparably frequent among ethnicities of African descent, while homozygous or compound heterozygous variants were most frequent in individuals of mixed ethnicity.

The finding that D negativity was due to a homozygous *RHD* gene deletion in approximately 50% of individuals of African descent and comparable between ethnic groups was unexpected, since it was previously shown that the D– status was associated with *RHD*08 N.01* and *RHD*03 N.01* alleles in approximately 66 and 15% of black African donors, repsecively.[4] This difference was due to the lower frequency of individuals with *RHD*08 N.01* alleles (20%) in our study.

TABLE 2. Ethnicity and *RHD* variants in D– pregnant Surinamese women and D– newborns			
Ethnicity	Number of individuals*	Number (%) of individuals with *RHD* variants	Number (%) of *RHD* variants[†]	Number and specificity of *RHD* alleles
Maroons	59	31 (53)	37 (31)	19 *RHD*03N.01*; 13 *RHD*08N.01*; 2 *RHD*09.01*; 1 *RHD*09.02*; 1 *RHD*09.03.01*; 1 *RHD*01N.20*
Creoles	28	13 (46)	16 (29)	9 *RHD*08 N.01*; 5 *RHD*03N.01*; 1 *RHD*10.00*; 1 *RHD*09.03.01*
Mixed	14	6 (43)	9 (32)	4 *RHD*03N.01*; 4 *RHD*08N.01*; 1 *RHD*01EL.01*
Hindustani	7	0	0	NA
Other/unknown	5	0	0	NA
Total	113	50 (44)	62 (26)	

* Excluded were the seven individuals with the *RHD*01/RHD*01 N.01* genotype, i.e., four creoles, one mixed, one Hindustani, and one other/unknown ethnicity.
† Denominator for calculating the percentage of *RHD* alleles per ethnicity was 118 for Maroons, 56 for creoles, and 28 for mixed ethnicity.
NA = not applicable.

Discrepancies in the molecular mechanism for D-negativity may, next to miscegenation in a multiethnic society, be explained by studying individuals from different parts of Africa, reflecting the genetic heterogeneity of *RHD* allele distributions among African populations.[13,14]

In Suriname, the D antigen is serologically determined using a single anti-D reagent, prohibiting the suspicion on *RHD* variants with weak D expression. Guidelines recommend the use of two selected anti-D reagents to determine the D-status, although DEL will not be detected and this strategy does not distinguish between partial and weak D.[2,15,16] The selection of anti-D reagents should be based on the prevalence and characterization of *RHD* variants in the population.[17] In our Surinamese cohort, the most clinically relevant variants (*RHD*09.01, RHD*09.02, RHD*09.03.01*) belonged to the weak D Type 4 cluster and should react weakly (i.e., less than 4+) with the majority of commercially available monoclonal anti-D's. When reactions with both reagents are negative an indirect antiglobulin test should be performed to detect very weak D expression. Weak reactions and discrepancies in reaction strength between the two reagents or methods determines further investigations. Subsequent *RHD* genotyping may provide clear guidance on anti-D preventive measures.

Women with partial (n = 4 in our study) and weak D, other than Types 1 to 3, should be classified as D– with regard to prenatal management and anti-D prophylaxis. Anti-D produced by women with *RHD* variants have been responsible for severe hemolytic disease of the fetus and newborn.[18–21] The woman of mixed ethnicity with the *RHD*01EL.01* variant in our study can be regarded not at risk for anti-D formation and anti-D prophylaxis is not necessary.[19,22,23] Although reports on the risk on primary immunization in D– pregnant women carrying fetuses with variant *RHD* are scarce but presumed low, anti-D prophylaxis, at least after delivery, should be administered.[7,8]

Our previous cross-sectional study showed that 12% of multiparous D– women had anti-D.[11] Eleven (13%) of the 84 women that were investigated in the current study had anti-D. A homozygous *RHD* deletion was present in five women, while six women had one or two *RHD*-null alleles. Treatment (i.e., phototherapy and exchange transfusions) for severe anti-D hemolytic disease was needed in two newborns from women with homozygous *RHD* deletions; the other nine newborns (of which seven D+ and two D-) did not need treatment.[24]

Our study was performed in D– pregnant women and D– newborns, while also in the transfusion situation serologically D– donors may carry *RHD* variants.[25,26] Although our cohort may not be representative for Surinamese donors, when we translate our findings to D– donors then potentially immunizing *RHD* variants would be present in approximately 7% (i.e., eight of our 120 individuals), underscoring the relevance of genotyping donors.

Our study had some limitations: First, our study was performed in D– individuals, while *RHD* variants are also common in serologically D+ individuals from African descent.[27] In the presence of variants, serologically D+ women may be at risk for anti-D when pregnant with children carrying wild-type D antigens.[28,29] Second, the *RH*-MLPA assay detects most common *RHD* variants and other (novel) variants may have been missed. Sequencing of the complete coding sequence may overcome this limitation. Last, in seven cases, discrepant results between serology (D–) and genotyping (wild-type *RHD*) were found, which may reflect errors in sample identification (the newborns from the three D+ mothers were serologically D+) or serologic testing problems. Unfortunately, blood to repeat serologic tests was not available. Rh-associated glycoprotein gene (RHAG) mutations that may result in disturbed D expression with normal *RHD* gene sequence were not investigated and cannot be excluded.[30,31]

In conclusion, *RHD* variants, in serologically D– Surinamese individuals, are common among ethnicities from African ancestry. However, the vast majority of our women with variants had *RHD*-null or partial *RHD* that would need anti-D prophylaxis when pregnant of a D+ child, implying that genotyping has limited benefit. Rather, genotyping will increase costs in women, given the low frequency of variants that do not need anti-D prophylaxis.[32] On the other hand, given the frequency of potentially immunizing *RHD* variants in D– newborns, genotyping these may be considered to provide guidance on anti-D preventive measures. However, this policy will be challenging in countries with limited financial resources, such as

Suriname. In the meantime adherence to international guidelines for the determination of the D status is recommended.

AUTHOR CONTRIBUTIONS

RZ, HHHK, WCWRZ, AB, and HS conceived and designed the study; AJ and BV performed and interpreted *RHD* variant genotyping; all authors acquired, analyzed, or interpreted the data; RZ, AJ, and HS drafted the manuscript; and all authors critically revised the manuscript for important intellectual content and gave final approval of the version to be published.

CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

TABLE S1. Ethnicity and *RHD* genotypes in serologically D− pregnant Surinamese women and D− newborns.
TABLE S2. Inheritance of *RHD* variant genes in the 11 mother-child combinations.
Fig. S1. *RHD* allele distribution in serologically D− pregnant Surinamese women and D− newborns of Maroon, Creole and Mixed ethnicity.