The Truth About RH Genotyping

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Background

For decades RHD genotyping has been performed by analysing a handful of single nucleotide polymorphisms (SNPs) resulting in a low resolution RHD genotype. Although this approach is feasible to discriminate the predominantly weak D variants type 1, 2 and 3 within the Caucasian population, **it lacks resolution in discriminating partial and hybrid variants found in other populations.** RHCE genotyping was even more limited in testing the basic SNPs distinguishing C/c and E/e, neglecting the presence of RHCE variants with missing high frequency antigens. **These ambiguous SNP test results translate to uncertainties regarding the RHD and RHCE phenotype, the prediction of the RHD-CE haplotype,** the transfusion policy and rhesus D prophylaxis. This makes a more accurate identification of RHCE (Van Sandt *et. al.* 2023) and RHD necessary as a standard of care, especially in people with broad allelic diversity such as Sub-Saharan Africans.

How accurate is RHD genotyping using SSP or exon-based NGS?

Selected cohort for RHD and RHCE

- RHD – RHCE hybrids, n=6
- Compound heterozygous RHD-RHCE variants (non hybrid), n=10
- Single RHD variants (non hybrid) , n=8

Comparison of 3 methods

- BAG Weak D and Partial D Type: SSP – gelelectrophoresis
- Inno-train CDE eXtend: elaborate SSP – qPCR TaqMan
- Inno-train NGS CORE: Short read NGS exon based - MiSeq

Results

1) The RHD-CE hybrids alleles tested in this cohort all had a different RHD genotyping result depending on the kit used. The results found per sample differ around the same position or region in the RHD gene, indicating the position of deviation compared to the reference RHD*01, see examples in table 1. The BAG SSP test package identifies RHD variants by testing a limited number of SNPs and missing amplicons compared to the reference RHD*01. CDE eXtend uses the same principle, but based on a more extensive SNP panel including extra variant SNPs and wild type (RHD*01) SNP positions which results in a better prediction of the RHD-CE hybrid alleles.
NGS CORE failed to identify 3 out of 4 of the different hybrid alleles tested due to missing exon data due to failed amplification and/or RHD-RHCE cross talk. To complete and correctly identify hybrid RH alleles long read NGS phasing all exons is needed.
→ RHD genotyping using SNPs or unphased exon sequencing is not suitable to identify RHD-CE hybrids.

2) Compound heterozygous RHD and/or CE (non-hybrid) variant allele combinations (n= 10) were correctly identified by both CDE eXtend and NGS CORE. The BAG test often resulted in no matching result for RHD, due to conflicting information of the combined variant alleles present. The added value of NGS CORE was to identify rare and new SNPs which were missed by SSP tests.
→ The correct identification of compound heterozygous SNP results requires a method that is able to detect/visualize the mutant and the wild type nucleotides.

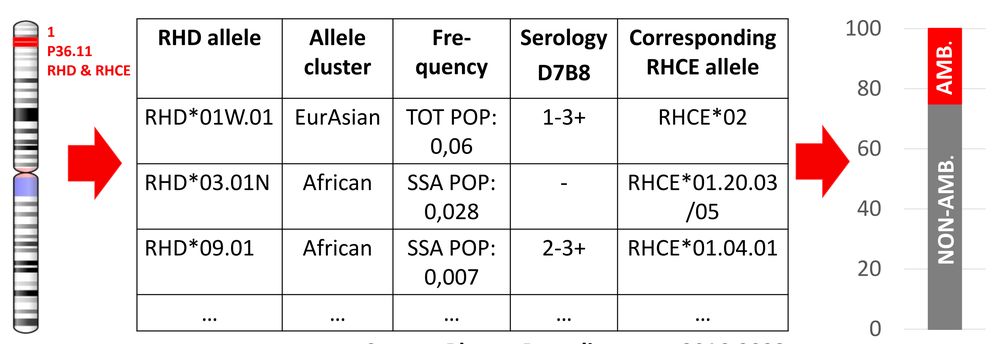
3) Single, non-hybrid RH variants, the easiest samples in this cohort (n=10), were correctly identified by both SSP tests.
→ Basic SNPs testing is sufficient for identification of simple, single RH variants.

The lack of SNP or exon phasing renders the SSP as well as the exon based NGS RHD genotype still 'very' ambiguous, often with conflicting data regarding the phenotype. In order to use this data in a clinical setting a sound method of narrowing down these ambiguities is necessary. An algorithm based on the patients origin, the allele frequency within that population, the predicted RHD-RHCE haplotype and the serology is used. This allows prediction of a reliable intermediate resolution RHD geno- and predicted RHD phenotype result in approximately 75% of the cases. 25% of the results remain ambiguous with more than one likely allele combination.
→ Origin RHD-RHCE haplotype matching reduces ambiguities.

Table 1: examples of RH hybrid results	Sample 1 Result	Sample 1 SNP	Sample 2 Result	Sample 2 SNP
WEAK D SSP BAG	RHD*01W.20	***E10*** *c1250T>C*	No weak D	N/A
PARTIAL D SSP BAG	RHD(1-9)-CE(10)	***E10*** *RHCE*	RHD*01N.05	***E3-7*** *CE*
CDE eXtend SSP Inno-train	Del: RHD*01EL.11	***E10*** *c1252-1253 ins T*	RHD*03N.01	***E4-7*** *CE with 733C>G*
NGS CORE Inno-train	Del: RHD*01EL.32	***E10*** *data missing in NGS result*	RHD*03N.01/ RHD*03N.02	***E4-7*** *CE Or* ***E4-9*** *CE with 733C>G*
Serology: Ortho D7B8	RhD NEG		RhD NEG	

It's a RHD E10 variant, but which one?

It's a RHD- RHCE hybrid, but which one?



Source: Rhesus Base, literature 2016-2023

Conclusions

These data demonstrate that primer design and test setup in SSP and NGS can lead to the misinterpretation of the RHD and RHCE genotyping results. It is therefore important to be aware of the limitations and remaining ambiguities of the test. **More complex RH samples with hybrids or combinations of RH variants need phased genotyping information such as Long Read NGS.**

Combining RHD and RHCE genotyping information, with serology, the allele's prevalence within the patients population and the predicted RHD-RHCE haplotype will allow to reduce ambiguities and assess the possible immunization risk.

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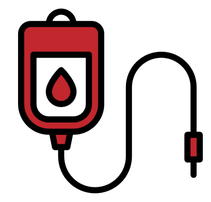

References:

Chang *et al.* 2020, Chou *et al.* 2018, Gaspardi *et al.* 2016, Keller *et al.* 2022, Kshitij Srivastava *et al.* 2016, Sippert *et al.* 2023, Van Sandt et al. 2023, Zang *et al.* 2022.

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