35th Regional Congress of the ISBT, 2025, Milan, Italy

Combined *RHD* and *RHCE* sequencing with long reads reveal novel alleles

M. Gueuning[1], G. A. Thun[1], S. Sigurdardottir[2], N. Trost[2], K. Engehausen[2], C. Noppen[3], C. Engström[4], S. Meyer[2]

[1] Blood Transfusion Service Zurich (SRC), Research and Development, Schlieren, Switzerland
[2] Blood Transfusion Service Zurich (SRC), Molecular Diagnostics and Cytometry, Schlieren, Switzerland
[3] Viollier AG, Allschwil, Switzerland
[4] Blood Transfusion Service Zurich (SRC), Immunohematology, Schlieren, Switzerland

Background
Long-read sequencing has proven reliable to resolve blood group alleles as full haplotypes. Here, we analyzed seven (I-VII) unresolved cases using combined *RHD* and *RHCE* sequencing by Oxford Nanopore Technologies (ONT). Five cases showed previously weakened antigen expression of either RH1, RH2 or RH4, but none of the most common causative variants were detected by routine genotyping methods. The remaining two cases were RhD-negative (RH:-1), with *RHD* gene positivity suggesting an underlying *RHD* null allele.

Aims
We used third generation ONT sequencing to clarify seven samples in which genotyping approaches had failed to explain the observed serological findings in the RH blood group system.

Methods
Standard serological techniques and commercially available PCR-SSP kits were applied to pheno- and genotype RH antigens. Long-range PCR was used to co-amplify the highly homologous *RHD* and *RHCE* genes with five generic primer pairs. Amplicon lengths ranged from 12.3 to 15.2 kb with at least 1.1 kb overlap between adjacent fragments to facilitate the phasing of variants on different amplicons. Samples were barcoded and sequenced on a MinION flow cell. Reads were mapped to the GRCh38 human genome and variants were called with Clair3. Sanger sequencing of the respective *RHD* or *RHCE* exon was used as confirmation method.

Results
Read distribution was even between as well as within samples resulting in a minimal depth of ~150 for the least covered fragment. We uncovered two novel *RHD* alleles with hitherto undescribed genetic variants, all present in a hemizygous state. In two samples (I & II), we found c.284A>G (p.Asp95Gly) in exon 2 (likely extracellular), which serologically resulted in no (I) or very weak (II) agglutination (1+) with anti-RH1. One case (III) depicted c.349A>G (p.Thr117Ala) in exon 3 with reduced antigen expression (3+ with different RH1 antibodies), which is in agreement with the predicted intracellular location of the variant. In another sample (IV), we detected an indel variant, c.395_396dupGG (p.Lys133GlyfsTer10), resulting in a frame shift and RhD negativity, which was confirmed by flow cytometry. This allele was previously described, but has not yet been included in the ISBT *RHD* allele table. With respect to *RHCE*, in one sample (V) we found a novel c.347C>A variant (p.Ala116Asp) in exon 3, which could be phased to the *RHCE*02* allele and explains the weak RH2 besides a normal RH4 expression. Novel is also a 3-bp duplication (c.341_343dupGGC; p.Arg114dup) that we found in a donor

(VI) whose red blood cells showed weak agglutination with anti-RH4. Indeed, this variant was phased to *RHCE*01*. Finally, we found c.19C>T (p.Arg7Trp) in exon 1 on *RHCE*01* in a heterozygous *RHCE*01*/*RHCE*02* sample (VII), explaining the weak reaction in serological tests with anti-RH4. This variant, although not reported in the ISBT *RHCE* allele table, has been detected with an allele frequency of ~2 per 10,000 in the general population (rs372501633). All variants were successfully confirmed by Sanger sequencing.

Summary/Conclusions

Using long-read sequencing, we resolved seven cryptic cases regarding the RH system among which five appeared as novel alleles. ONT sequencing in combination with long-range PCR proved suitable to resolve challenging alleles with the particular asset to phase variants. Rather than deducing the affected allele by making use of the phenotype data, sequencing by ONT allowed us to formally phase the observed variant to the proper *RHCE*01* or *RHCE*02* background whenever appropriate.