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Rh Blood Group D Antigen Genotyping Using a Portable Nanopore-based Sequencing Device: Proof of Principle

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BACKGROUND: Nanopore sequencing is direct sequencing of a single-stranded DNA molecule using biological pores. A portable nanopore-based sequencing device from Oxford Nanopore Technologies (MinION) depends on driving a DNA molecule through nanopores embedded in a membrane using a voltage. Changes in current are then measured by a sensor, thousands of times per second and translated to nucleobases.

METHODS: Genomic DNA (gDNA) samples ($n = 13$) were tested for Rh blood group D antigen (*RHD*) gene zygosity using droplet digital PCR. The *RHD* gene was amplified in 6 overlapping amplicons using long-range PCR. Amplicons were purified, and the sequencing library was prepared following the 1D Native barcoding gDNA protocol. Sequencing was carried out with 1D flow cells R9 version. Data analysis included basecalling, aligning to the *RHD* reference sequence, and calling variants. Variants detected were compared to the results acquired previously by the Ion Personal Genome Machine (Ion PGM).

RESULTS: Up to 500× sequence coverage across the *RHD* gene allowed accurate variant calling. Exonic changes in the *RHD* gene allowed *RHD* allele determination for all samples sequenced except 1 *RHD* homozygous sample, where 2 heterozygous *RHD* variant alleles are suspected. There were 3 known variant *RHD* alleles (*RHD*01W.02*, *RHD*11*, and *RHD*15*) and 6 novel *RHD* variant alleles, as previously seen in Ion PGM sequencing data for these samples.

CONCLUSIONS: MinION was effective in blood group genotyping, provided enough sequencing data to achieve high coverage of the *RHD* gene, and enabled confident calling of variants and *RHD* allele determination.

Introduction

Nanopore sequencing, also known as third-generation sequencing (TGS) and single-molecule sequencing (SMS), enables fast and direct sequencing of single-stranded DNA molecules using biological pores. Nanopore sequencing, first proposed in the 1980s (1, 2), overcomes limitations in sequencing by synthesis technologies, such as next-generation sequencing (NGS), and allows faster library preparation and real-time sequence data analysis. Although NGS has allowed for high throughput sequencing while lowering the cost, short reads generated during NGS library preparation have made de novo assembly for large genomes difficult due to repetitive DNA sequences (3–5). TGS does not rely on PCR amplification but aims for SMS with real-time data analysis. The PCR-free approach in TGS abolishes sequencing biases introduced by PCR (6, 7). The advancement of TGS reduced time of library preparation and sequencing from days to hours when compared to NGS (3, 7).

In 2014, Oxford Nanopore Technologies introduced the small portable nanopore-based sequencing device, named the MinION (8, 9), which offered different cost-efficient sequencing kits to meet various sequencing needs. MinION sequencer technology is based on a flow cell containing 512 pores that are derived from *Escherichia coli* curli (2, 10), embedded in a synthetic membrane submerged in ionic solution (7, 8). By applying a voltage, a DNA molecule is driven through the pores causing changes in the ionic current running through the pores in a distinctive manner, described as "squiggle" (2, 7). These changes are measured by a sensor thousands of times per second (11), which are then translated to nucleotides using software, in a process known as basecalling.

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MinION, when first introduced, had a 65% to 88% accuracy rate (3, 12). Recent advancement in sequencing chemistry and computational software, however, reduced the error rate to 5% to 15% (2). Sequencing yield and accuracy, when used for whole microbial genome sequencing, has reached 97% for 2D chemistry experiments and 94% for 1D experiments (13).

The MinION sequencer has been used in infectious agent surveillance and clinical diagnosis since these areas would benefit the most from real-time sequencing technology (3). Studies have shown the great potential of the MinION, for example, during the Ebola and Zika virus outbreaks (14, 15). The technology was also used to sequence the SARS-CoV-2 during the COVID-19 pandemic (16–18). Different studies have used the nanopore sequencer to detect DNA and RNA modification, such as methylation in bacterial and mammalian genomes (19–21).

Although the use of nanopore sequencing has not been widely investigated in blood group genotyping, it has been shown effective for clinical genotyping of human leukocyte antigens (22–24). Other real-time SMS technology has been used to genotype the *ACKR1* gene that encodes the Duffy blood group antigens, resulting in the establishment of *ACKR1* allele-specific reference sequences (25). MinION has also been used in *ABO* genotyping (26) by sequencing a 7 kb amplicon, covering the region of exons 6 and 7, successfully allowing the differentiation of 6 ABO genotypes.

The Rh blood group system (ISBT004) is the second most important blood group system after ABO (27, 28) and 1 of the most polymorphic blood group systems. The Rh protein is expressed in a complex transmembrane structure, where the RhCcEe protein is encoded by the *RHCE* gene and the RhD protein is encoded by the *RHD* gene (29, 30). The D antigen is the most clinically significant antigen in the Rh system due to its high immunogenicity and being the main cause of hemolytic disease of the fetus and newborn.

In 2018, the *RHD* gene was fully sequenced using NGS (31) on the Ion Personal Genome Machine (Ion PGM) where allele-specific reference sequences were established. In this study, we tested the suitability and efficiency of the MinION sequencer in blood group genotyping by fully sequencing the *RHD* gene and comparing the results to *RHD* genotyping results obtained previously using Ion PGM (31, 32).

Materials and Methods

SAMPLE PROCESSING

One blood donor sample (National Health Service Blood and Transplant, Bristol, UK) and 12 genomic DNA (gDNA) samples from Finnish pregnant females phenotyped as RhD negative, supplied by Finnish Red Cross Blood Service (Helsinki, Finland) with full ethical approval, were genotyped for the *RHD* gene and results were published in 2020 (32). The serology testing performed on the samples from the Finnish pregnant females included a red cell antibody adsorption and elution test for the *RHD* blood group DEL phenotype to determine if there were a low number of D antigens per red cell (32).

The blood donor sample was serologically phenotyped for different blood group antigens by the National Health Service Blood and Transplant. The sample was received in an EDTA tube, which was centrifuged at 2500*g* for 10 min at room temperature. The plasma on the top layer was carefully disposed, and buffy coat was collected into a 1.5 mL tube; the remaining content was discarded. gDNA was extracted from buffy coat using the QIAamp DNA Blood Mini kit (Qiagen Ltd) as previously described (31).

All samples were tested to determine *RHD* gene zygosity (hemizygous [Dd] or homozygous [DD]) using droplet digital PCR. Samples were tested for 2 targets on the *RHD* gene, exon 5 and exon 7, against the reference gene *AGO1* on chromosome 1 (33, 34).

The *RHD* gene was amplified from gDNA samples in 6 overlapping amplicons using previously described primers (31). Amplicons were then purified using Agencourt AMPure XP reagent (Beckman Coulter) and then pooled in a quantitative manner to ensure equal representation of each amplicon, yielding a final amount of 1800 ng in 48 µL final volume.

LIBRARY PREPARATION AND SEQUENCING

Sequencing library was prepared following the 1D Native barcoding gDNA protocol using the Native Barcoding kit 1–12 and the Ligation Sequencing kit (SQK-LSK109) with 1D flow cells R9 version (Oxford Nanopore Technologies). A flow cell was placed in the MinION, which was then plugged directly into a USB3 port on a laptop running Windows 10. MinKNOW v1.13 software (Oxford Nanopore Technologies) was connected to the MinION, and the software ran default control checks on the quality of the sequencing pores. The flow cell was primed as per manufacturer guidelines, and the sequencing library was then added. In the MinKNOW v1.13 software, the sequencing run was started and left running for 12 h. Raw signal data (FAST5 files) were then transferred to an external hard drive for analysis.

DATA ANALYSIS

The sequencing run produced 49 FAST5 files, each containing about 4000 reads. Guppy basecaller v3.2.4 (Oxford Nanopore Technologies) was used for basecalling the raw data (FAST5), which divided the read

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into pass and fail FASTQ files by comparing the quality score per read to a threshold ≥ 7 (35). Only pass reads were used to carry on the analysis. Files were subjected to sequencing quality analysis using EPI2ME software (Oxford Nanopore Technologies).

FASTQ files were concatenated for barcoding using Samtools v.1.4.1. Porechop software v.0.2.1 was used for barcoding, which divided reads by barcodes. Barcoded reads were then trimmed using SeqKit software v0.7.1. A script was written in Bash to automate the process of the analysis. Nanopolish software v.0.9.0 was used to index the reference human genome build 38 (hg38) chromosome 1 reference sequence (NC_000001.11), index the FASTQ files, and then map the reads to the reference, which generated BAMfiles. BAMfiles were then sorted using Samtools v.1.4.1 to generate BAI files. Variants were then called using Nanopolish software v.0.9.0 (36). The reads were visualized using Integrative Genomics Viewer v.2.5.3 (Broad Institute and the Regents of the University of California) and CLC Main Workbench 10 software (Qiagen Ltd). Variant calling was also performed using CLC MainWorkbench 10 software at 100× minimum coverage. The data were then compared to the sequencing data obtained from Ion PGM. Exonic and intronic mutations detected from both platforms were compared, and the variant tracks of the same sample were aligned for comparison.

Results

All samples were tested for *RHD* gene zygosity using droplet digital PCR with all samples having a hemizygous *RHD* gene (1 copy), except for 2 samples that showed a homozygous *RHD* gene (2 copies). Samples (n = 13) (Table 1) were serologically phenotyped as RhD-negative or weak D by serology. The *RHD* gene was fully sequenced using the MinION sequencer using overlapping long-range PCR (LR-PCR) amplicons. Quality assessment for MinION reads was performed, and a mean quality PHRED score of 11 was detected and reads length mode of 10 450 bp. PHRED quality score is an algorithmic integer value representing the estimated probability of an error in the identification of a base. A score of 10 indicates a 1/10 probability of an incorrect base or a 90% confidence in the called base.

Data were analyzed and mapped to the *RHD* hg38 reference sequence, which was visualized using Integrated Genome Viewer software. As noted previously (31), the *RHD* human reference sequence in the hg38 encodes a variant *RHD* allele *RHD*DAU0* encoded by c.1136C>T (p.Thr379Met) in exon 9. Therefore, all 13 samples showed a homozygous single nucleotide polymorphism (SNP) in exon 9 c.1136T>C (Met379Thr) (31).

Variant calling was performed, and a variant track was generated for each sample, which was then compared to the variant track generated from the Ion PGM sequencing data for the same sample. All exonic SNPs detected in the 13 samples agreed with the ones detected from the Ion PGM data (Table 1). The *RHD* allele was determined in all samples sequenced except for one *RHD* homozygous sample where the results were inconclusive. In this sample, 4 heterozygous mutations were detected (c.48G>C, c.602C>G, c.667T>G, c.819G>A) (Table 1), suggesting the presence of a wild-type *RHD* allele, which did not agree with the weak D serology result; thus, genotyping results remained inconclusive.

Intronic changes detected were also compared and agreed with the SNPs detected by Ion PGM (31), except for 6 SNPs. These 6 intronic SNPs were expected to be specific to the *RHD* reference sequence hg38 *RHD*DAU0*, which included 25 286 520 T>C, 25 286 601 T>A, 25 286 605 A>T, 25 286 674 C>T, 25 286 732 A>G, and 25 295 850 A>G and were mainly located in intron 2. These SNPs were most probably false-positive SNPs from the Ion PGM data assembly of the short reads that were generated during library preparation.

Discussion

In this study, SMS was used through MinION sequencing for *RHD* genotyping. Thirteen samples were sequenced, and the results were compared to the ones obtained by Ion PGM. *RHD* gene genotyping using MinION proved to be successful, and alleles determined agreed with the ones identified using NGS (Ion PGM). The *RHD* gene from the 13 samples was sequenced, and the *RHD* allele was determined for all samples except for one where the presence of 2 *RHD* variant alleles is expected. Two samples showed the same novel variant (Val141fs/Val141Glu), but we confirmed that these samples were from 2 separate individuals.

In the sample where sequencing was inconclusive, determined to be *RHD* homozygous by droplet digital PCR, 4 heterozygous exonic SNPs were detected including c.48G>C in exon 1, c.602C>G in exon 4, c.667T>G in exon 5, and c.819G>A in exon 6 (Table 1), which indicated the presence of 2 variant *RHD* alleles (compound heterozygote) (32). Allele phasing was not possible because PCR amplicons were used for sequencing. Possible alleles encoded by these exonic changes are either *RHD*09.03.01* (encoded by c.602C>G, c.667T>G, c.819G>A) and *RHD*01.01* (encoded by c.48G>C; considered wild-type) or *RHD*09.04* (encoded by c.48G>C, c.602C>G, c.667T>G, c.819G>A) and *RHD*01* (considered wild-type) (see online Supplemental Fig. 1). However, the presence of a wild-type *RHD* allele that produced

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Table 1 RHD alleles identified in samples sequenced using MinION.							
Rh serology	RhD phenotype	*RHD* zygosity	SNPs[b]	Exon	amino acid	*RHD* allele	Reference
CcEE[a]	Weak D+	Homozygous	—[c]	—[c]	—[c]	*RHD*01*	NA[d]
Ccee	Negative	Hemizygous	885G>T	6	Met295Ile	*RHD*11*	(40)
Ccee	Negative	Hemizygous	845G>A	6	Gly282Asp	*RHD*15*	(40)
Ccee	Weak D+	Homozygous	48G>C	1	Trp16Cys	Undetermined[e]	NA
			602C>G	4	Thr201Arg		
			667T>G	5	Phe223Val		
			819G>A	6	silent		
Ccee	Negative	Hemizygous	829G>A	6	Gly277Arg	Novel variant	MN365996 (32)
Ccee	Negative	Hemizygous	784delC	5	Gln262fs	Novel variant	MN365997 (32)
Ccee	Negative	Hemizygous	421delG	3	Val141fs	Novel variant	MN365995 (32)
			422T>A	3	Val141Glu		
Ccee	Negative	Hemizygous	421delG	3	Val141fs	Novel variant	MN365995 (32)
			422T>A	3	Val141Glu		
CcEe	Negative	Hemizygous	1154G>C	9	Gly385Ala	*RHD*01W.02*	(40)
ccEe	Negative	Hemizygous	1154G>C	9	Gly385Ala	Novel variant	MN365998 (32)
			1163T>G	9	Leu388Arg		
ccEe	Negative	Hemizygous	519C>G	4	Tyr173Stop	Novel variant	MN365999 (32)
ccEe	Negative	Hemizygous	845G>A	6	Gly282Asp	*RHD*15*	(40)
ccEe	Weak D+	Hemizygous	1016G>C	7	Gly339Ala	Novel variant	MN366002 (32)

[a]Blood donor sample (National Health Service Blood and Transplant).
[b]SNPs detected by MinION support the results detected by Ion PGM published in 2020 (32).
[c]No exonic SNPs detected.
[d]Not applicable.
[e]Undetermined due to the presence of possibly 2 *RHD* variant alleles.

a normal RhD protein would not agree with the weak D reactivity in serology, as normal D would mask the weak D reactivity, and the result would be RhD positive instead of weak D. Since the presence of an intact copy of either *RHD*01.01* or *RHD*01* allele is unlikely, genotyping results for this sample remain inconclusive. It is possible that the seemingly wild-type copy of the *RHD* gene carried a deletion that was concealed by the presence of an intact copy of the mutated *RHD* gene (either *RHD*09.03.01* or *RHD*09.04*). Due to the location of the *RHD* primers, variation in the promotor of the *RHD* gene cannot be ruled out for this sample. Variation in the Rh-associated glycoprotein (*RHAG*) gene for this sample can also not be excluded. Only DNA was available from this sample, and so no mRNA sequencing could be performed from either cultured red cells or reticulocytes.

The advantages of using MinION over NGS are the faster library preparation, real-time sequencing analysis, and sequencing of longer reads that allow for better assembly (3, 6, 36). In this study, MinION library preparation and sequencing time was reduced to 1 day compared to 4 days for NGS, considering that library preparation started after the LR-PCR amplification and purification, which takes 3 days for 20 samples. The bioinformatics for basecalling and determination of variants with the MinION sequencing takes approximately 1 to 2 days. Although LR-PCR amplicons were used to amplify the *RHD* gene for sequencing, direct sequencing of any target gene is the main goal with SMS. One prior study (37) employed target enrichment using biotinylated PCR-generated baits that allowed capturing the targeted gene for MinION sequencing.

An error in the MinION sequencing occurs in specific sequences with an estimated 11% error rate (38). MinION, with 40× depth of coverage, may cause a false substitution and insertion every 10 to 50 kb and a false deletion every 1000 bp, which may cause an issue in detection of variations (38). According to a prior study (12), the MinION error rate per base with a certain quality number does not correspond to the error rate per base expected for the PHRED value of the same quality results in MinION technology. Even though the MinION

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sequencing quality score does not correspond with the PHRED score used for NGS technologies, it is still used as an error estimation score. Using R6 MinION chemistry, MinION had an approximately 40% error rate on single-read sequencing (12). In our study, however, the current work 1D flow cells R9 version was used for sequencing, which showed a lower error rate (13).

We did not encounter any issue in calling variants since high coverage across the gene was achieved with up to 500× coverage in some regions. Exonic and intronic SNPs were detected, and alleles were determined, which agreed with ones found using NGS (Ion PGM). Variation in coverage is expected because multiple LR-PCR amplicons are sequenced. Eliminating the need for PCR amplification should speed the library preparation process and enable allele phasing. This might be possible through targeted MinION sequencing using Cas9 guided adaptors ligation (39) or biotinylated PCR (38). Eliminating the PCR amplification step should enable easier allele phasing, which is important in blood group genotyping to enable assigning alleles successfully in hemizygous samples and identifying novel deletions, insertions, or hybrid alleles.

Other challenges facing SMS are data handling, storage, and analysis. The evolving nature of this sequencing technology makes it difficult to establish a user-friendly software that would enable fast and accurate data analysis to make it suitable for clinical use. Currently, there are numerous published reports about the utilization of MinION and data handling and analysis. Most of these papers, however, focused on genome assembly and analysis for microorganisms (14, 15). The human genome is larger and far more complex; therefore, more work is needed to explore the potential power of this approach in human genome sequencing and analysis to improve sequencing accuracy and develop user-friendly interfaces for data analysis (38).

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: TGS, third-generation sequencing; SMS, single-molecule sequencing; NGS, next-generation sequencing; Ion PGM, Ion Personal Genome Machine; gDNA, genomic DNA; hg38, Genome Reference Consortium human build 38 GRCH38); LR-PCR, long-range PCR; SNPs, single nucleotide polymorphisms.

Human Genes: *RHD*, Rh blood group D antigen; *RHCE*, Rh blood group CcEe antigens; *ABO*, ABO, alpha 1-3-N-acetylgalactosaminyltransferase and alpha 1-3-galactosyltransferase; *ACKR1*, atypical chemokine receptor 1 (Duffy blood group); *AGO1*, argonaute RISC component 1; *RHAG*, Rh associated glycoprotein.

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W.A. Tounsi wrote manuscript; W.A. Tounsi performed experiments; W.A. Tounsi and V.P. Lenis analyzed data; S.M. Tammi, S. Sainio, and K. Haimila collected and processed Finnish samples; T.E. Madgett and N.D. Avent supervised study and revised manuscript. All authors reviewed, edited, and approved the manuscript.

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