2022-06-02

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10.1093/clinchem/hvac075
Clinical Chemistry

This is a pre-copyedited, author-produced version of an article accepted for publication in Clinical Chemistry following peer review. The version of record hvac075 is available online at: https://academic.oup.com/clinchem/advance-article/doi/10.1093/clinchem/hvac075/6598034 and https://doi.org/10.1093/clinchem/hvac075

Rh blood group D antigen genotyping using a portable nanopore based sequencing device: proof of principle

Running header: ***RHD* genotyping using nanopore based sequencing**

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Abbreviations:

genomic DNA (gDNA)

polymerase chain reaction (PCR)

Ion Personal Genome Machine (Ion PGM)

third generation sequencing (TGS)

single-molecule sequencing (SMS)

next-generation sequencing (NGS)

blood group genotyping (BGG)

National Health Service Blood and Transplant (NHSBT)

droplet digital PCR (ddPCR)

human genome build (hg38)

long-range PCR (LR-PCR)

single nucleotide polymorphisms (SNPs)

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

Abstract

BACKGROUND:

Nanopore sequencing is direct sequencing of a single stranded DNA molecule using biological pores. A portable nanopore based sequencing device (MinION) from Oxford Nanopore Technologies 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 polymerase chain reaction (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 500x 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 one *RHD* homozygous sample, where two heterozygous *RHD* variant alleles are suspected. There were three known variant *RHD* alleles (*RHD*01W.02, RHD*11 and RHD*15*) and six 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.

Keywords

RHD genotyping, blood group genotyping, MinION sequencing, third generation sequencing, single molecule sequencing, nanopore, Rh blood group, D negative

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 polymerase chain reaction (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 flowcell 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.

MinION, when first introduced, had a 65-88% accuracy rate (3, 12). Recent advancement in sequencing chemistry and computational software, however, reduced the error rate to 5-15% (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 (BGG), 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 atypical chemokine receptor 1 *(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, alpha 1-3-N-acetylgalactosaminyltransferase and alpha 1-3-galactosyltransferase (*ABO*) genotyping (26) by sequencing a 7 kb amplicon, covering the region of exons 6 and 7, successfully allowing the differentiation of six ABO genotypes.

The Rh blood group system (ISBT004) is the second most important blood group system after ABO (27, 28) and one 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 Rh blood group CcEe antigens (*RHCE*) gene

and the RhD protein is encoded by the Rh blood group D antigen (*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 BGG 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 (NHSBT), Bristol, United Kingdom) 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 where there are a low number of D antigens per red cell (32).

The blood donor sample was serologically phenotyped for different blood group antigens by the NHSBT. The sample was received in an ethylenediaminetetraacetic acid tube, which was centrifuged at 2500g for 10 minutes 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, United Kingdom) as previously described (31).

All samples were tested to determine *RHD* gene zygosity (hemizygous (Dd) or homozygous (DD)) using droplet digital PCR (ddPCR). Samples were tested for two targets on the *RHD* gene, exon 5 and exon 7, against the reference gene Argonaute RISC Component 1 *(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, United Kingdom) and then pooled in a quantitative manner, to ensure equal representation of each amplicon, to yield 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, UK). 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) 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) was 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 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 visualised using Integrative Genomics Viewer v.2.5.3 (Broad Institute and the Regents of the University of California, United States) and CLC Main Workbench 10 software (Qiagen Ltd, United Kingdom). Variant calling was also performed using CLC MainWorkbench 10 software at 100x minimum coverage. The data was 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 ddPCR with all samples having a hemizygous *RHD* gene (one copy), except for two samples that showed a homozygous *RHD* gene (two 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, for example, a score of 10 indicates a 1/10 probability of an incorrect base or a 90% confidence in the called base.

Data was analysed and mapped to the *RHD* hg38 reference sequence, which was visualised 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 SNP in exon 9 c.1136T>C (Met379Thr) (data not shown, 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 single nucleotide polymorphisms (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; 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 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 13 samples, was sequenced and the *RHD* allele was determined for all samples except for one where the presence of two *RHD* variant alleles is expected. Two samples showed the same novel variant (Val141fs/Val141Glu) but we confirmed that these samples were from two separate individuals.

In the sample where sequencing was inconclusive, determined to be *RHD* homozygous by ddPCR, 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 two variant *RHD* alleles (compound heterozygote) (32). Allele phasing was not possible due to the fact that 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) (Supplemental Figure 1). However, the presence of a wild type *RHD* allele that produced 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 a day compared to 4 d for NGS, considering that library preparation started after the LR-PCR amplification and purification, which takes 3 d for 20 samples. The bioinformatics for base calling and determination of variants with the MinION sequencing takes ~1-2 d. 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 40x depth of coverage, may cause a false substitution and insertion every 10–50 kb and a false deletion every 1000 bp, which may cause an issue in detection of variations (38). According to Laver (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 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 ~40% error rate on single read sequencing (12). In our study here, 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 500x 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 due to the fact that 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 BGG 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 utilisation 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).

Authors contribution: W.A.T wrote manuscript; W.A.T performed experiments; W.A.T and V.P.L analysed data; S.M.T, S.S, and K.H collected and processed Finnish samples; T.E.M. and N.D.A. supervised study and revised manuscript. All authors reviewed, edited and approved the manuscript.

Conflict-of-interest disclosure: N.D.A. is a consultant for Natera Inc. and has an expert testimony appointment with Wilmer-Hale.

Funding: The research was funded by King Abdulaziz University, Jeddah, Saudi Arabia.

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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[e]
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 602C>G 667T>G 819G>A	1 4 5 6	Trp16Cys Thr201Arg Phe223Val silent	Undetermined[d]	NA[e]
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 422T>A	3 3	Val141fs Val141Glu	Novel variant	MN365995 (32)
Ccee	Negative	Hemizygous	421delG 422T>A	3 3	Val141fs Val141Glu	Novel variant	MN365995 (32)
CcEe	Negative	Hemizygous	1154G>C	9	Gly385Ala	*RHD*01W.02*	(40)
ccEe	Negative	Hemizygous	1154G>C 1163T>G	9 9	Gly385Ala Leu388Arg	Novel variant	MN365998 (32)
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 (NHSBT).

[b]SNPs detected by MinION support the results detected by Ion PGM published in 2020 (32).

[c]No exonic SNPs detected.

[d]Undetermined due to the presence of possibly two *RHD* variant alleles.

[e]NA; Not applicable.