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

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4   **Rh blood group D antigen genotyping using a portable nanopore based**

5   **sequencing device: proof of principle**

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7   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:

3

76 MinION was effective in blood group genotyping, provided enough sequencing data

77 to achieve high coverage of the *RHD* gene and enabled confident calling of variants

78 and *RHD* allele determination.

79

80 **Keywords**

83

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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.

109   MinION, when first introduced, had a 65-88% accuracy rate (3, 12). Recent

110   advancement in sequencing chemistry and computational software, however,

111   reduced the error rate to 5-15% (2). Sequencing yield and accuracy, when used for

112   whole microbial genome sequencing, has reached 97% for 2D chemistry

113   experiments, and 94% for 1D experiments (13).

114   The MinION sequencer has been used in infectious agent surveillance and

115   clinical diagnosis since these areas would benefit the most from real-time

116   sequencing technology (3). Studies have shown the great potential of the MinION,

117   for example, during the Ebola and Zika virus outbreaks (14, 15). The technology was

118   also used to sequence the SARS-CoV-2 during the COVID-19 pandemic (16 - 18).

119   Different studies have used the nanopore sequencer to detect DNA and RNA

120   modification, such as methylation in bacterial and mammalian genomes (19 - 21).

121   Although the use of nanopore sequencing has not been widely investigated in

122   blood group genotyping (BGG), it has been shown effective for clinical genotyping of

123   human leukocyte antigens (22- 24). Other real-time SMS technology has been used

124   to genotype the atypical chemokine receptor 1 *(ACKR1)* gene that encodes the Duffy

125   blood group antigens, resulting in the establishment of *ACKR1* allele specific

126   reference sequences (25). MinION has also been used in ABO, alpha 1-3-N-

127   acetylgalactosaminyltransferase and alpha 1-3-galactosyltransferase (*ABO*)

128   genotyping (26) by sequencing a 7 kb amplicon, covering the region of exons 6 and

129   7, successfully allowing the differentiation of six ABO genotypes.

130   The Rh blood group system (ISBT004) is the second most important blood

131   group system after ABO (27, 28) and one of the most polymorphic blood group

132   systems. The Rh protein is expressed in a complex transmembrane structure, where

133   the RhCcEe protein is encoded by the Rh blood group CcEe antigens (*RHCE*) gene

134 and the RhD protein is encoded by the Rh blood group D antigen (*RHD*) gene (29,

135 30). The D antigen is the most clinically significant antigen in the Rh system due to

136 its high immunogenicity and being the main cause of hemolytic disease of the fetus

137 and newborn

138   In 2018, the *RHD* gene was fully sequenced using NGS (31) on the Ion

139 Personal Genome Machine (Ion PGM) where allele specific reference sequences

140 were established. In this study, we tested the suitability and efficiency of the MinION

141 sequencer in BGG by fully sequencing the *RHD* gene and comparing the results to

142 *RHD* genotyping results obtained previously using Ion PGM (31, 32).

143

144 **Materials and Methods**

145 *Sample processing*

146   One blood donor sample (National Health Service Blood and Transplant

147 (NHSBT), Bristol, United Kingdom) and 12 genomic DNA (gDNA) samples from

148 Finnish pregnant females phenotyped as RhD negative, supplied by Finnish Red

149 Cross Blood Service (Helsinki, Finland) with full ethical approval, were genotyped for

150 the *RHD* gene and results were published in 2020 (32). The serology testing

151 performed on the samples from the Finnish pregnant females included a red cell

152 antibody adsorption and elution test for the *RHD* blood group DEL phenotype where

153 there are a low number of D antigens per red cell (32).

154   The blood donor sample was serologically phenotyped for different blood

155 group antigens by the NHSBT. The sample was received in an

156 ethylenediaminetetraacetic acid tube, which was centrifuged at 2500g for 10 minutes

157 at room temperature. The plasma on the top layer was carefully disposed and buffy

158 coat was collected into a 1.5-mL tube; the remaining content was discarded. gDNA

159   was extracted from buffy coat using the QIAamp DNA Blood Mini kit (Qiagen Ltd,

160   United Kingdom) as previously described (31).

161   All samples were tested to determine *RHD* gene zygosity (hemizygous (Dd) or

162   homozygous (DD)) using droplet digital PCR (ddPCR). Samples were tested for two

163   targets on the *RHD* gene, exon 5 and exon 7, against the reference gene Argonaute

164   RISC Component 1 *(AGO1)* on chromosome 1 (33, 34).

165   The *RHD* gene was amplified from gDNA samples in 6 overlapping amplicons

166   using previously described primers (31). Amplicons were then purified using

167   Agencourt AMPure XP reagent (Beckman Coulter, United Kingdom) and then pooled

168   in a quantitative manner, to ensure equal representation of each amplicon, to yield a

169   final amount of 1800 ng in 48 µL final volume.

170

171   *Library preparation and sequencing*

172   Sequencing library was prepared following the 1D Native barcoding gDNA

173   protocol using the Native Barcoding kit 1-12 and the Ligation Sequencing kit (SQK-

174   LSK109) with 1D flow cells R9 version (Oxford Nanopore Technologies, UK). A Flow

175   cell was placed in the MinION, which was then plugged directly into a USB3 port on

176   a laptop running Windows 10. MinKNOW v1.13 software (Oxford Nanopore

177   Technologies) connected to the MinION and the software ran default control checks

178   on the quality of the sequencing pores. The flow cell was primed as per

179   manufacturer guidelines and the sequencing library was then added. In the

180   MinKNOW v1.13 software, the sequencing run was started and left running for 12 h.

181   Raw signal data (FAST5 files) was then transferred to an external hard drive for

182   analysis.

183

184 *Data analysis*

185   The sequencing run produced 49 FAST5 files each containing about 4000

186 reads. Guppy basecaller v3.2.4 (Oxford Nanopore Technologies) was used for

187 basecalling the raw data (FAST5), which divided the read into pass and fail FASTQ

188 files by comparing the quality score per read to a threshold ≥ 7 (35). Only pass reads

189 were used to carry on the analysis. Files were subjected to sequencing quality

190 analysis using EPI2ME software (Oxford Nanopore Technologies).

191   FASTQ files were concatenated for barcoding using Samtools v.1.4.1.

192 Porechop software v.0.2.1 was used for barcoding, which divided reads by barcodes.

193 Barcoded reads were then trimmed using SeqKit software v0.7.1. A script was

194 written in Bash to automate the process of the analysis. Nanopolish software v.0.9.0

195 was used to index the reference human genome build 38 (hg38) chromosome 1

196 reference sequence (NC_000001.11)), index the FASTQ files, and then map the

197 reads to the reference which generated BAMfiles. BAMfiles were then sorted using

198 Samtools v.1.4.1 to generate BAI files. Variants were then called using Nanopolish

199 software v.0.9.0 (36). The reads were visualised using Integrative Genomics Viewer

200 v.2.5.3 (Broad Institute and the Regents of the University of California, United

201 States) and CLC Main Workbench 10 software (Qiagen Ltd, United Kingdom).

202 Variant calling was also performed using CLC MainWorkbench 10 software at 100x

203 minimum coverage. The data was then compared to the sequencing data obtained

204 from Ion PGM. Exonic and intronic mutations detected from both platforms were

205 compared and the variant tracks of the same sample were aligned for comparison.

206

207 **Results**

208   All samples were tested for *RHD* gene zygosity using ddPCR with all samples

209   having a hemizygous *RHD* gene (one copy), except for two samples that showed a

210   homozygous *RHD* gene (two copies). Samples (n=13) (Table 1) were serologically

211   phenotyped as RhD negative or weak D by serology. The *RHD* gene was fully

212   sequenced using the MinION sequencer using overlapping long-range PCR (LR-

213   PCR) amplicons. Quality assessment for MinION reads was performed and a mean

214   quality PHRED score of 11 was detected and reads length mode of 10,450 bp.

215   PHRED quality score is an algorithmic integer value representing the estimated

216   probability of an error in the identification of a base, for example, a score of 10

217   indicates a 1/10 probability of an incorrect base or a 90% confidence in the called

218   base.

219   Data was analysed and mapped to the *RHD* hg38 reference sequence, which

220   was visualised using Integrated Genome Viewer software. As noted previously (31),

221   the *RHD* human reference sequence in the hg38 encodes a variant *RHD* allele

222   *RHD*DAU0* encoded by c.1136C>T (p.Thr379Met) in exon 9. Therefore, all 13

223   samples showed a homozygous SNP in exon 9 c.1136T>C (Met379Thr) (data not

224   shown, 31).

225   Variant calling was performed and a variant track was generated for each

226   sample, which was then compared to the variant track generated from the Ion PGM

227   sequencing data for the same sample. All exonic single nucleotide polymorphisms

228   (SNPs) detected in the 13 samples agreed with the ones detected from the Ion PGM

229   data (Table 1). The *RHD* allele was determined in all samples sequenced except for

230   one *RHD* homozygous sample where the results were inconclusive. In this sample, 4

231   heterozygous mutations were detected (c.48G>C, c.602C>G, c.667T>G, c.819G>A)

232   (Table 1), suggesting the presence of a wild type *RHD* allele, which did not agree

233   with the weak D serology result, thus genotyping results remained inconclusive.

234   Intronic changes detected were also compared and agreed with the SNPs

235   detected by Ion PGM (31), except for 6 SNPs. These 6 intronic SNPs were expected

236   to be specific to the *RHD* reference sequence hg38 *RHD*DAU0*, which included

237   25,286,520 T>C; 25,286,601 T>A; 25,286,605 A>T; 25,286,674 C>T; 25,286,732

238   A>G; 25,295,850 A>G and were mainly located in intron 2. These SNPs were most

239   probably false positive SNPs from the Ion PGM data assembly of the short reads

240   that were generated during library preparation.

241

242   **Discussion**

243   In this study, SMS was used through MinION sequencing for *RHD* genotyping.

244   Thirteen samples were sequenced and results were compared to the ones obtained

245   by Ion PGM. *RHD* gene genotyping using MinION proved to be successful and

246   alleles determined agreed with the ones identified using NGS (Ion PGM). The *RHD*

247   gene, from 13 samples, was sequenced and the *RHD* allele was determined for all

248   samples except for one where the presence of two *RHD* variant alleles is expected.

249   Two samples showed the same novel variant (Val141fs/Val141Glu) but we

250   confirmed that these samples were from two separate individuals.

251   In the sample where sequencing was inconclusive, determined to be *RHD*

252   homozygous by ddPCR, 4 heterozygous exonic SNPs were detected including

253   c.48G>C in exon 1, c.602C>G in exon 4, c.667T>G in exon 5, and c.819G>A in exon

254   6 (Table 1), which indicated the presence of two variant *RHD* alleles (compound

255   heterozygote) (32). Allele phasing was not possible due to the fact that PCR

256   amplicons were used for sequencing. Possible alleles encoded by these exonic

11

257   changes are either *RHD*09.03.01* (encoded by c.602C>G, c.667T>G, c.819G>A)

258   and *RHD*01.01* (encoded by c.48G>C; considered wild type) or *RHD*09.04*

259   (encoded by c.48G>C, c.602C>G, c.667T>G, c.819G>A) and *RHD*01* (considered

260   wild type) (Supplemental Figure 1). However, the presence of a wild type *RHD* allele

261   that produced a normal RhD protein would not agree with the weak D reactivity in

262   serology, as normal D would mask the weak D reactivity and the result would be

263   RhD positive instead of weak D. Since the presence of an intact copy of either

264   *RHD*01.01* or *RHD*01* allele is unlikely, genotyping results for this sample remain

265   inconclusive. It is possible that the seemingly wild type copy of the *RHD* gene carried

266   a deletion that was concealed by the presence of an intact copy of the mutated *RHD*

267   gene (either *RHD*09.03.01* or *RHD*09.04*). Due to the location of the *RHD* primers,

268   variation in the promotor of the *RHD* gene cannot be ruled out for this sample.

269   Variation in the Rh Associated Glycoprotein (*RHAG*) gene for this sample can also

270   not be excluded. Only DNA was available from this sample and so no mRNA

271   sequencing could be performed from either cultured red cells or reticulocytes.

272   The advantages of using MinION over NGS are the faster library preparation,

273   real-time sequencing analysis and sequencing of longer reads that allow for better

274   assembly (3, 6, 36). In this study, MinION library preparation and sequencing time

275   was reduced to a day compared to 4 d for NGS, considering that library preparation

276   started after the LR-PCR amplification and purification, which takes 3 d for 20

277   samples. The bioinformatics for base calling and determination of variants with the

278   MinION sequencing takes ~1-2 d. Although LR-PCR amplicons were used to amplify

279   the *RHD* gene for sequencing, direct sequencing of any target gene is the main goal

280   with SMS. One prior study (37) employed target enrichment using biotinylated PCR-

281   generated baits that allowed capturing the targeted gene for MinION sequencing.

282   An error in the MinION sequencing occurs in specific sequences with an

283 estimated 11% error rate (38). MinION, with 40x depth of coverage, may cause a

284 false substitution and insertion every 10–50 kb and a false deletion every 1000 bp,

285 which may cause an issue in detection of variations (38). According to Laver (12),

286 the MinION error rate per base with a certain quality number does not correspond to

287 the error rate per base expected for the PHRED value of the same quality results in

288 MinION technology. Even though the MinION sequencing quality score does not

289 correspond with the PHRED-score used for NGS technologies, it is still used as an

290 error estimation score. Using R6 MinION chemistry, MinION had an ~40% error rate

291 on single read sequencing (12). In our study here, however, the current work 1D flow

292 cells R9 version was used for sequencing, which showed a lower error rate (13).

293   We did not encounter any issue in calling variants since high coverage across

294 the gene was achieved with up to 500x coverage in some regions. Exonic and

295 intronic SNPs were detected and alleles were determined, which agreed with ones

296 found using NGS (Ion PGM). Variation in coverage is expected due to the fact that

297 multiple LR-PCR amplicons are sequenced. Eliminating the need for PCR

298 amplification should speed the library preparation process and enable allele phasing.

299 This might be possible through targeted MinION sequencing using Cas9 guided

300 adaptors ligation (39) or biotinylated PCR (38). Eliminating the PCR amplification

301 step should enable easier allele phasing, which is important in BGG to enable

302 assigning alleles successfully in hemizygous samples and identifying novel

303 deletions, insertions or hybrid alleles.

304   Other challenges facing SMS are data handling, storage and analysis. The

305 evolving nature of this sequencing technology makes it difficult to establish a user-

306 friendly software that would enable fast and accurate data analysis to make it

307   suitable for clinical use. Currently, there are numerous published reports about the

308   utilisation of MinION and data handling and analysis. Most of these papers, however,

309   focused on genome assembly and analysis for microorganisms (14, 15). The human

310   genome is larger and far more complex; therefore, more work is needed to explore

311   the potential power of this approach in human genome sequencing and analysis to

312   improve sequencing accuracy and develop user friendly interfaces for data analysis

313   (38).

314

315   **Authors contribution:** W.A.T wrote manuscript; W.A.T performed experiments;

316   W.A.T and V.P.L analysed data; S.M.T, S.S, and K.H collected and processed

317   Finnish samples; T.E.M. and N.D.A. supervised study and revised manuscript. All

318   authors reviewed, edited and approved the manuscript.

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429   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)

430   [a]blood donor sample (NHSBT).

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

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432   [c]No exonic SNPs detected.

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

434   [e]NA; Not applicable.