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The MinION as a cost-effective technology for diagnostic screening of the *SCN1A* gene in epilepsy patients

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ABSTRACT

The MinION is a portable DNA sequencer that allows real time sequencing at low capital cost investment. We assessed accuracy and cost-effectivess of the MinION for genetic diagnostic testing of known *SCN1A* mutations that cause Dravet Syndrome (DS). DNA samples (n = 7) from DS patients previously shown to carry *SCN1A* mutations via Ion Torrent and Sanger sequencing were sequenced using the MinION. *SCN1A* amplicons for 8 exons were sequenced using the MinION with 1D chemistry on an R9.4 flow cell. All known missense mutations were detected in all samples showing 100 % concordance with results from other methods. However, the MinION failed to detect the insertions/deletions (INDELs) present in these patients. Nevertheless, these results indicate that MinION is a cost-effective platform for use as an initial screening step in the detection of nucleotide substitution mutations in in *SCN1A*, especially in under-resourced laboratories or hospitals. Further improvements are required to reliably detect INDELS in this gene.

1. Introduction

Dravet Syndrome (DS), first described as severe myoclonic epilepsy of infancy (SMEI) and later classified as epileptic encephalopathy, early infantile 6, is a rare and severe form of epileptic encephalopathy. It begins in the first year of life with recurrent seizures triggered by fever in infants (Dravet, 2011). DS is a highly drug-resistant epilepsy that can often be difficult to diagnose (Connolly, 2016). Although development is normal at onset, over time, multiple seizure types, mainly myoclonic, atypical absences, and focal seizures appear, as well as developmental delay and accompanying cognitive and behavioral disorders (Dravet, 2011; Depienne et al., 2009). Seizure types can be focal or generalized, including high risk for status epilepticus (SE), which are a threat to life. These seizure types can result in sudden unexpected death in epilepsy (SUDEP) and all contribute toward a significantly increased mortality rate in DS (Cooper et al., 2016; Connolly, 2016; Auvin et al., 2018).

In 2001, Claes et al., reported that mutations in the Sodium Voltage-Gated Channel Alpha Subunit 1 gene (*SCN1A)* were responsible for causing DS, where 70–80 % of DS patients were found to carry a heterozygous mutation(s) in the *SCN1A* gene, with ~90 % of these cases arising *de novo* (Claes et al., 2001). The gene that encodes the alpha-1 subunit of the sodium channel (denoted Nav1.1), plays an important role in controlling the excitability of neurons (Claes et al., 2001). Early diagnosis of DS is important to identify the best treatment options for patients in order to avoid specific anti-seizure medications that may exacerbate their seizures, as well as aggressive seizure control that may improve developmental outcomes (Tomonoh et al., 2015; Ziobro et al., 2018).

Genetic testing can help confirm a clinical diagnosis, provide useful prognostic information regarding the natural history of the disorder increase treatment options and allow for specific genetic counselling (Hirose et al., 2013; Dunn et al., 2018). Sanger sequencing (SS) and Next

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Generation Sequencing (NGS) technologies are major methodological procedures that can be used to detect *SCN1A* mutations (Usluer et al., 2016; Brunklaus et al., 2013). NGS, also known as massively parallel sequencing, is a powerful technology characterized by the ability to perform high throughput sequencing of millions of reads to sequence single genes up to the entire genome in a relatively inexpensive manner. Despite the increased utility of NGS, it requires a high initial cost for instrumentation, and as such, this can be a limitation for small local research centers and hospitals(Kchouk et al., 2017; Dunn et al., 2018).

Oxford Nanopore Technologies (ONT) has released a handheld sequencing device called the MinION through the MinION Access Program (MAP), which was deployed extensively for sequencing bacterial and viral genomes (Loman et al., 2016; Votintseva et al., 2017). The MinION is a portable long-read sequencer that allows real time sequencing and is connected to a PC or laptop through a high-speed USB cable. During sequencing, a single strand of DNA is analysed through a membrane via a nanopore by an applied electric field (Mikheyev and Tin, 2014). More importantly, MinION provides sequencing reagents and two flow cells for only ~USD1000 as part of a starter pack. MinION long-read sequencing has already been successfully used to detect mutations in *CYP2D6*, *HLA-A*, and *HLA-B* (Torti et al., 2015), *TP53* in cancer (Tota et al., 2016), *ABL1* in leukemia (Albano et al., 2017) and *GBA* in Parkinson's and Gaucher (Mokretar et al., 2019).

Here, we evaluate the potential of the MinION for clinical diagnostics by sequencing *SCN1A* exons to detect DS-specific mutations and assess accuracy and cost-effectiveness by comparing results with another NGS method (Ion Torrent) and to gold standard Sanger sequencing.

2. Methods

2.1. Patients and DNA samples

Epileptic patients were diagnosed specifically with DS by their clinical Neurologists and via detection of *SCN1A* mutations previously identified by Targeted gene panel through the use of Ion Torrent PGM and/or SS. DNA samples were available from seven patients who carried exonic *SCN1A* mutations and were included in this study to validate MinION. Six patients possessed missense mutations and two patients possessed INDELs, with one patient possessing two different mutations – (see Supplementary Information Table 1). DNA was extracted from peripheral blood with the purity ranging from 1.82 to 1.96 at the 260/280 ratio using Nanodrop Spectrophotometer 8000.

The targeted NGS panel including *SCN1A* sequencing has been previously reported (Maksemous et al., 2016). Library preparation was performed using the Ion AmpliSeq library kit 2.0 (Thermo Fisher Scientific, Scoresby, Victoria, Australia) as previously described (Maksemous et al., 2016). Template preparation performed on the Ion PGM OT2 200 Template Kit (Thermo Fisher Scientific, Scoresby, Victoria, Australia), according to the manufacturers' instructions (part no. 4480974 Rev. 4.0) (Maksemous et al., 2016). Sequencing was performed on the Ion Torrent Personal Genome Machine (PGM) system using Ion Sequencing 200 Kit V2 and an Ion 316 Chip (Thermo Fisher Scientific, Scoresby, Victoria, Australia) according to the manufacturers' procedures (Cat. no.4482006 Rev.1.0). Sanger sequencing was performed using the ThermoFisher BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Scoresby, Victoria, Australia) on the Applied Biosystems™ 3500 Series Genetic Analyzer (Thermo Fisher Scientific, Scoresby, Victoria, Australia).

2.2. Barcoding, library preparation and sequencing

A library was prepared using the PCR Barcoding Kit (SQK-PBK004, up to 12 barcodes) according to the Four-primer PCR protocol from ONT. We used previously reported primers to amplify eight exons of *SCN1A* containing previously detected mutations in the patient DNA samples (Claes et al., 2001). However, the universal tail sequences provided by Oxford Nanopore Technology (forward primer: 5′-TTCTGTTGGTGCTGATATTGC-3′, reverse primer: 5′-ACTTGCCTGTCGCTCTATCTTC-3′) were added to the primers (Supplementary information Table 2), primers were then reordered from the Integrated DNA Technologies (IDT) (Baulkham Hills, NSW, Australia). Following amplification, all samples were re-quantified using a Qubit 2.0 Fluorometer (Life Technologies). Samples were then diluted to a concentration of 20 ng/μl and used as templates (20 ng) for the PCR barcoding step. The PCR reaction mix contained 0.5μM forward primer, 0.5μM reverse primer, 10 μL Epicentre MasterAmp buffer E, 0.2 μL Taq polymerase (PromegaGoTaq), 7.8 μL nuclease free water and 1 μL sample DNA. Based on the manufacturer's protocol, PCR cycling condition were as follows: Stage 1, 94 °C for 1 min, 5 cycles of 94 °C for 1 min, 60 °C for 1 min, 65 °C for 1 min; Stage 2, 30 cycles of 94 °C for 30 s, 62 °C for 1 min, 65 °C for 1 min, Stage 3, 65 °C for 5 min, and hold 4 °C. Gel images indicated that the four-primer protocol was not producing an amplified product, so the PCR step was repeated using our own protocol, with thermal cycling conditions has changed: 94 °C for 4 min., 35cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min. and 4 °C hold. Amplicons were isolated and purified using Agencourt AMPure XP magnetic beads, quantified using dsDNA HS assay kit on a Qubit 2.0 Fluorometer, and pooled to an equal weight ratio. The equal ratio of all sample libraries were then multiplexed in one flow cell run. MinION sequencing was performed using R9.4 flow cell (FLO-MIN106, ONT) according to the manufacturer's instructions and run for 48 h. MinION sequencing was controlled using ONT MinKNOW software.

2.3. Data analysis

Real-time analysis of the sequence files generated by MinION was performed using a custom script including Fast5 extraction. Base calling of the raw MinION data was performed with the cloud-based software (Metrichor, http://www.metrichor.com), generating Fast5 files, from which Fastq were extracted and demultiplexed with LAST-version 959 (https://www.protocols.io/view/demultiplexing-nanopore-reads-with-last-xj3fkqn). The basecaller divides reads in two folders "fail" and "pass", only "pass" reads were used in this study.

We performed analysis of MinION sequencing data with bioinformatics method including read mapping, variant calling and annotation with the samtools/bcftools package. Single nucleotide variants (SNV) and insertions/deletions (INDELs) detection were called in VCF files. Reads were aligned to the GRCh37 human reference genome, using NM_006920.4 as a reference, and visualized with the Integrative Genomics Viewer (IGV) browser (Thorvaldsdóttir et al., 2013; Robinson et al., 2011). All BAM and VCF files generated are available as supplementary data for researchers wishing to conduct further analyses.

3. Results

In this study we used the MinION to sequence DNA samples from seven DS patients who had previously been shown to carry exonic *SCN1A* mutations ie. six patients with single nucleotide variants (SNVs) and two patients with INDELs nb. one patient had two different mutations. Previously designed primers were used to target eight exons in SCN1A and custom PCR protocols were developed prior to sequencing runs.

3.1. Detection of SNVs

The MinION sequencing of DS patients detected all missense and synonymous variants previously detected by Sanger Sequencing and Ion Torrent PGM. The average sequencing depth of SNVs was $>=$ 40x coverage. The accuracy ranged from 90 to 100 % (median = 99 %) (Table 1).

Table 1
SNVs detected by MinION and Sanger/NGS sequencing.

Subject ID	Barcode	Variant	Type	Amino Acid Change	Depth	Accuracy	Zygosity
DGR 308	BC 01	c.1048A > T	Missense	p.Met350Leu	40	100	Het
DGR 310	BC 03	c.4063 G > A	Missense	p.Val1355Ile	114	96.0	Het
DGR 311	BC 04	c.2803C > T	Missense	p.Arg935Cys	267	90	Het
DGR 312	BC 05	c.2653 G > T	Missense	p.Val885Phe	126	100	Het
		c.2856 T > C	Synon	p.(=)	322	74.9	Het
DGR 316	BC 09	c.4839 G > A	Synon	p.(=)	56	99.21	Het
DGR 317	BC 10	c.1354A > T	Missense	p.Lys452Ter	121	99.2	Het
		c.1212A > G	Synon	p.(=)	60	100	Hom

The zygosity in which they were detected, is shown (het = heterozygous, hom = homozygous) GenBank reference sequence NM_006920.4.

3.2. Detection of INDELS

Two exonic INDELs detected by Sanger and/or NGS were not called by MinION ie. c.703_703delA/p.Thr235fs, c.4449_4449delA/p.Ile1483Metfs*18. To better understand the potential sources of variant calling errors, we reviewed the position of these INDELs.

For c.703_703delA in sample DGR 308, the variation is at the end of a group of four A nucleotides and the failure to detect this variant may be the result of slippage of the amplicon in the pore, leading to an erroneous number of bases being counted. While the Ion Torrent also has some problems with homopolymer regions and is subject to slippage, the c.703_703delA variant was confirmed by Sanger sequencing. Similarly, for c.4449_4449delA in sample DGR 313, the variation is between a group of four G nucleotides, and amplicon slippage may have interfered with quality of reads at this location sufficient to prevent detection without visualization of the region. Software improvements in the basecaller (e.g. run-length encoding) may improve this in the future for already-sequenced reads. ONT has also recently commercialised an R10.3 flow cell with an extended read head that can more easily quantify longer homopolymer sequences (up to 10bp).

3.3. Cost-effectiveness of MinION testing

The MinION has a low initial capital investment cost, with starter packs that includes the MinION device, sequencing reagents and two flow cells for only $1000US.

At the time of writing, we estimated the cost of using the MinION at around $291US per sample for sequencing all *SCN1A* exons, when multiplexing 12 samples. The amount of labor time including sample sequencing and data analysis, consumable supplies, and equipment utilized are shown in Supplementary Information Table 3, which were determined through direct observation of testing procedures. The flow cell is included in the cost, using one flow cell for $900US for 12 barcoded samples ($75US/sample). The labor/time required to run a 12-sample experiment is 1−2 days which is comparable to Illumina (Table 2). All software programs used for data analysis are freely available. There's no additional cost required for their use. There is some labor cost to set up a bioinformatics pipeline, but the marginal cost for variant detection after the pipeline has been set up is fairly low.

The currently available tests for whole *SCN1A* exons sequencing are $368 US using an Ion Torrrent NGS custom panel (diagnostic 5-gene panel, Genomics Research Center), $552US for Illumina based sequencing and $2070 for Sanger sequencing. At "Prevention Genetics" the cost for sequencing *SCN1A* using an exome-based NGS approach is $640US (preventiongenetics.com) (Table 2). Further reduction of MinION costs can be achieved when purchasing the flow cells in a pack of 48 ($500US). Furthermore, the MinION flow cell can be washed and reused by following the manufacturer's protocol. ONT have also released smaller-scale, cheaper flow cells called Flongles, which cost approximately 1/10 the price of the MinION flow cell, i.e. $90 USD (https://store.nanoporetech.com/flongle.html). Flongle cells are intended to provide a faster and more cost-effective sequencing system for smaller tests (yield up to 500MB is reasonable), and may be suitable for at least a pre-screen of *SCN1A*. Costs could be further reduced via the application of more samples per flow cell. ONT have a PCR barcoding kit that allows for up to 96, which provides enough for 10 libraries of 96 barcodes each. ONT have also released a BC13−24 kit native barcoding kit which allows for 6 libraries of an additional 12 barcodes each, allowing up to 24 per run via ligation.

Table 2
Time and cost comparison among sequencing platforms.

Method	Lab work duration /12 samples	Cost (USD)*/sample
MinION	1−2 days	$291
Ion Torrent	2−3 days	$368
Illumina	1−2 days	$552
Sanger	1−2 weeks	$2070

* USD = United States Dollars.

4. Discussion

The MinION is a portable and inexpensive sequencing device that generates long DNA sequence reads in real time. Compared with second generation sequencing platforms (e.g. Illumina and Ion Torrent), the advantages of Nanopore sequencing include the very low capital cost, space requirements, and turnaround time of analysis. The ONT sequencing technology has been successfully applied to studies in th fields of clinical microbiology, human genome sequencing and cancer cytotyping (Laver et al., 2015; Jansen et al., 2015).

The accurate and early diagnosis of DS will lead to avoidance of medications that may exacerbate seizures (Steel et al., 2017). Thus, *SCN1A* sequence analysis is essential for DS patients for routine diagnostic testing and improved patient point of care. Here, we analyzed 7 DNA samples from DS patients using MinION 1D chemistry on an R9.4 flow cell to detect *SCN1A* mutations, including 8 already identified mutations (6 SNVs and 2 INDELs). Variants were called using samtools/bcftools on data aligned with LAST version 959. We have attached the VCF output mapping to NM_006920.4. We found that the four-primer PCR protocol provided by ONT was inefficient and we had to use a previously optimized protocol. The four-primer protocol is known to be quite sensitive. This is possibly due to the binding dynamics involved in the PCR process, where undesired templates are synthesised (wasting nucleotides), and thus there is competition between the ONT primers and the custom primers binding to the target gene sequence region. It's possible that adjusting the relative concentrations of the primers could mitigate this competition although we recommend our custom protocol for users of this technology when aiming to detect these specific *SCN1A*mutations.

Although our analysis was only performed with one MinION run, we achieved > 40× coverage on average. Results were compared with SS and NGS (Ion Torrent), and our data indicates that MinION accurately detected all SNVs in all samples at the correct zygosity. However, the MinION failed to detect the two small INDELs previously detected by Ion Torrent and Sanger sequencing after variant calling.

ONT sequencing has gained enormous yield and accuracy over the

past few years. The per base accuracy of the MinION has been reported as 85–95 %, whereby the error rate is dominated by INDEL calls. This is consistent with our study which yielded an average accuracy of 99 % for SNVs, but failed to detect the INDELs. The MinION has a known bias for slippage in repeat regions and the INDELs were adjacent to homopolymer sequences. A limitation of the MinION is to accurately resolve homopolymers and detect small INDELs. Such anomalies are known to be the main errors in whole genome sequencing using the MinION (Sedlazeck et al., 2018; Jain et al., 2018). Of the 625 ClinVar reported pathogenic variants in *SCN1A* for all forms of epilepsy, the majority (439) are SNVs. 130 of the remaining variants and INDELs of 10bp or less, comprising 20.8 % of known epilepsy mutations. As the ability to detect these mutations is limited in the present MinION method, this does represent a limitation that needs to be overcome before MinION sequencing can be a comprehensive mutation screening tool for DS.

Nanopore is an emerging technology and based on the rapid developments in the flow cell chemistry, base calling algorithms and bioinformatics, we expect improvements in calling of small INDELs and further reduction of false positive SNV calls in the near future. Other long read single-molecule platforms are also being developed in order to overcome the limitations of the nanopore error rate to detect very low-level mosaic mutations. While it is estimated that mosaic, pathogenic *SCN1A* mutations are found in 1.3%–7.5% of DS patients, there appears to be a correlation of increased mutant allele fraction with severity of disease (Mei et al., 2019; Stosser et al., 2018). Furthermore, most low-level mosaic mutations of *SCN1A* are associate with asymptomatic or mildly affected parents of DS patients, although there are rarely some severely affected mosaic carriers of *SCN1A* mutations (Mei et al., 2019).

Despite the small cohort size and use of a single run, our results carry significant implications for developing a cost-effective technology for genetic diagnostic testing of DS patients on a routine basis. This will likely have an important impact on DS patient diagnosis and treatment. Whilst this study focused on sequencing the *SCN1A* gene, future work using the MinION technology could implement a multi-gene gene panel approach. The MinION system is able to generate up to 30Gb of sequencing reads which is sufficient to allow for an increased number of genes, from a targeted gene panel test (Orsini *et al.,* 2018) to even whole genome/whole exome sequencing (https://nanoporetech.com/products/minion). However, this may come with additional costs and turn-around time for variant curation and confirmation. Despite these limitations, a multi-gene panel would be particularly useful as other studies have shown that early onset epilepsy syndromes can often have different mutated genes (Symonds et al., 2019; Dunn et al., 2020).

Finally, this study was limited to testing the MinION for detection of known *SCN1A* mutations whereby we knew in advance the mutations possessed by each patient. When utilising this technology in a diagnostic laboratory practitioners can have high confidence of exonic SNV calls. However, we recommend that the gold standard SS approach also be implemented to confirm a molecular diagnosis of the patient sample. This might not be feasible for under-resourced laboratories in which case results should be interpreted with care. Also, failure to detect small INDELs does not exclude their presence although we expect improvements by ONT to overcome this problem. Also, for discovery of larger deletions and duplications material may need to be referred for further validation with other techniques such as multiplex ligation-dependent probe amplification, qPCR or competitive PCR. More stringent bioinformatics QC methods may also be used to help to increase the sensitivity of the MinION for mutation detection.

5. Conclusion

This is the first study using MinION to screen *SCN1A* for mutations in DS patients, which is one of the most severe epilepsy syndromes of early childhood. This study demonstrated that MinION is an effective diagnosis tool to screen for detection of *SCN1A* mutations in DS patients and supports it's routine use in the clinic. MinION is an attractive option for genetic diagnostic testing in under-resourced hospital settings and could also complement existing NGS technologies currently in use in genome diagnostic laboratories. With the rapid development of nanopore technology, in terms of chemistry advances, more accurate base callers and bioinformatics software, the MinION offers promise for a wide use in the future for routine use in diagnostic laboratories.

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