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Metagenomic arbovirus detection using MinION nanopore sequencing

Jana Batovska[a,b,*], Stacey E Lynch[a], Brendan C Rodoni[a,b], Tim I Sawbridge[a,b], Noel OI Cogan[a,b]

[a] Agriculture Victoria Research, AgriBio Centre for AgriBioscience, 5 Ring Road, Bundoora, Victoria, 3083, Australia
[b] School of Applied Systems Biology, La Trobe University, Bundoora, Victoria, 3086, Australia

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ABSTRACT

With its small size and low cost, the hand-held MinION sequencer is a powerful tool for in-field surveillance. Using a metagenomic approach, it allows non-targeted detection of viruses in a sample within a few hours. This study aimed to determine the ability of the MinION to metagenomically detect and characterise a virus from an infected mosquito. RNA was extracted from an *Aedes notoscriptus* mosquito infected with Ross River virus (RRV), converted into cDNA and sequenced on the MinION. Bioinformatic analysis of the MinION reads led to detection of full-length RRV, with reads of up to 2.5 kb contributing to the assembly. The cDNA was also sequenced on the MiSeq sequencer, and both platforms recovered the RRV genome with > 98% accuracy. This proof of concept study demonstrates the metagenomic detection of an arbovirus, using the MinION, directly from a mosquito with minimal sample purification.

1. Introduction

Metagenomic next-generation sequencing (NGS) allows for the unbiased detection of organisms within a sample. This powerful approach has enhanced pathogen detection for both diagnostic and surveillance applications (Barzon et al., 2011; Temmam et al., 2014). Until recently, NGS was restricted to the laboratory due to the size of the sequencers available; however, the release of the MinION (Oxford Nanopore Technologies) enables NGS in-field. The MinION is a low-cost, hand-held sequencer that produces long reads (up to 233 kb) in real-time (Jansen et al., 2017). At time of writing, the cost of a starter kit that includes the sequencer is USD $1000, with additional kits and flow cells ranging from USD $500 to $900. The sequencing accuracy is considerably lower than popular short-read sequencers such as the Illumina MiSeq and HiSeq, with an error rate of approximately 5–10% (Tyson et al., 2017) for 2D R9 reads, compared to < 0.1% for the Illumina sequencers (Houldcroft et al., 2017). However, multi-fold genome coverage can be used to generate an accurate consensus sequence, with the MinION achieving > 99% accuracy post-data analysis (Wang et al., 2015). Furthermore, low quality reads can often be adequate to identify a known pathogen (Walter et al., 2016), allowing rapid detection with more accurate characterisation performed later.

The MinION has been used to detect a variety of viruses, including Ebola (Hoenen et al., 2016; Quick et al., 2016), dengue (Mongan et al., 2015), Zika (Quick et al., 2017), influenza (Eckert et al., 2016; Wang et al., 2015), Flock House (Jaworski and Routh, 2017), and cowpox (Kilianski et al., 2015). The MinION has a lower throughput than NGS platforms such as Illumina (Lu et al., 2016), so these studies targeted the virus using PCR amplicons or hybridisation capture. This approach limits detection to known pathogens with characterised genome sequences. Despite a lower throughput, the MinION has been used to sequence Ebola, chikungunya, and hepatitis C viruses using an unbiased metagenomic approach with randomly amplified cDNA (Greninger et al., 2015). Kilianski et al. (2016) also used a metagenomic approach when sequencing Ebola virus and Venezuelan equine encephalitis virus with the MinION, however unamplified RNA/cDNA-hybrids were used for library preparation. Both of these studies indicate the MinION is sensitive enough to detect viruses without target enrichment. While all of the viruses sequenced using nanopore technology have been from clinical samples or viral cultures, the MinION has yet to be used to detect viruses directly from a viral vector, such as a mosquito.

Aedes albopictus and *Ae. aegypti* are key vectors of medically important arboviruses such as dengue, chikungunya and Zika (Patterson et al., 2016). As invasive species, they readily colonise new territories (Medlock et al., 2012; Zhong et al., 2013) and have been implicated in importing arboviruses into non-endemic areas (Lindsay et al., 2015). In efforts to prevent their establishment, biosecurity surveillance programs have included exotic mosquito monitoring at high-risk international ports for early detection (Knope et al., 2016; Medlock et al., 2012; Vaux and Medlock, 2015). Exotic mosquito surveillance relies on morphological identification of collected specimens, and is often complemented by molecular approaches, such as PCR, to confirm mosquito

* Corresponding author at: Agriculture Victoria Research, AgriBio Centre for AgriBioscience, 5 Ring Road, Bundoora, Victoria, 3083, Australia.
E-mail addresses: jana.batovska@ecodev.vic.gov.au (J. Batovska), stacey.lynch@ecodev.vic.gov.au (S.E. Lynch), brendan.rodoni@ecodev.vic.gov.au (B.C. Rodoni), tim.sawbridge@ecodev.vic.gov.au (T.I. Sawbridge), noel.cogan@ecodev.vic.gov.au (N.O. Cogan).

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species or screen for specific arboviruses. These approaches can take several days and delay appropriate control measures. The MinION can bring molecular testing of invasive species to the port of entry, and identify vector species and arbovirus carriage in real-time.

The aim of this study was to determine if MinION nanopore sequencing could be used to detect an arbovirus from a single mosquito using a metagenomic approach. In order to provide the most utility in biosecurity situations, the ability to detect the species of the mosquito was also tested. The performance of the MinION in this study was directly compared to the commonly used, lab-based MiSeq.

2. Materials and methods

2.1. Nucleic acid extraction and cDNA synthesis

A single *Ae. notoscriptus* mosquito infected with Ross River virus (RRV) strain QML1 (Jones et al., 2010) was used as the test sample. Rearing and infection of the mosquito is described in Batovska et al. (2017). The mosquito was homogenised in 180 µL of viral lysis buffer (Buffer AVL, Qiagen) using a TissueLyser (Qiagen) at 30 Hz and 2 cycles of 1 min. The homogenate was centrifuged (5 min, 10,000 rpm), and 140 µL of the supernatant was used for viral RNA purification using the QIAamp Viral RNA Mini Kit (Qiagen). The kit was used according to the manufacturer's instructions, however no carrier RNA was added to the Buffer AVL. The purified RNA was eluted in 80 µL and DNase treated using a TURBO DNA-free Kit (Ambion) according to manufacturer's instructions.

The RNA extract was quantified by a 2200 TapeStation using the RNA ScreenTape assay (Agilent Technologies). The quantification resulted in 10 µL of RNA being used as input for first-strand cDNA synthesis. The RNA was reverse transcribed with the SuperScript III First-Strand Synthesis System (Invitrogen) using 100 ng of random hexamers. Second strand cDNA synthesis was performed with the NEBNext mRNA Second Strand Synthesis Module (New England BioLabs). The cDNA was purified using AMPure XP beads (Beckman Coulter) with a 1.8 x beads ratio, quantified by a Qubit 1.0 Fluorometer (Life Technologies), and stored at −20 °C.

2.2. Quantification of viral load

The viral load of the mosquito was quantified using droplet digital PCR (ddPCR). The primer pair RRVE2F and RRVE2R, and RRVE2 Probe were used to amplify and detect RRV (Hall et al., 2011). The reaction mixture consisted of 12 µL of 2 x ddPCR Supermix for Probes (No dUTP) (Bio-Rad), 0.12 µL of each 100 µM/L primer, 0.06 µL of 100 µM/L probe, 0.03 µL of 100,000 U/mL HindIII-HF (New England Biolabs), 10.7 µL of water, and 6.25–200 pg of cDNA. The reaction mixture was partitioned into droplets using the Automated Droplet Generator loaded with oil for probes (Bio-Rad). After droplet generation, PCR was performed using the following conditions: 95 °C for 10 min; 40 cycles of 94 °C for 30 s and 60 °C for 1 min; and 98 °C for 10 min. A 2 °C/sec ramp rate was used for each step. The droplets were then analysed with a QX200 Droplet Reader (Bio-Rad) and QuantaSoft™ Analysis Pro software was used to provide the number of RRV cDNA copies per µL of the ddPCR reaction. A positive and negative control was used in the quantification reaction to verify results.

2.3. Nanopore library preparation and sequencing

Libraries were prepared with the SQK-NSK007 Sequencing Kit (R9 version) using the Oxford Nanopore Technologies (ONT) 2D cDNA sequencing protocol. The undiluted cDNA was A-tailed using the NEBNext Ultra II End Repair/dA-Tailing Module (New England BioLabs). The incubation periods were extended to 30 min to improve A-tailing. Adapters (ONT) were ligated onto the cDNA, and PCR was performed using LongAmp *Taq* DNA Polymerase (New England BioLabs), primers (ONT) and a 10 min 65 °C extension step. The amplified cDNA was again A-tailed using the method specified above, and ONT hairpin adapters and tethers were ligated onto the cDNA. Library purification was performed using Dynabeads MyOne Streptavidin C1 beads (Invitrogen) with a 0.5 x beads ratio, and a 30 min incubation at room temperature. The eluted library was quantified by a Qubit, and sequenced on a primed MinION Spot-ON Flow Cell Mk I (R9 version). The NC_48Hr_Sequencing_Run_FLO_MAP103 program was run on MinKNOW with local basecalling, thereby producing 1D reads. The use of 1D reads was chosen to determine if the field-appropriate Rapid 1D Sequencing Kit would be adequate for virus detection.

2.4. Illumina library preparation and sequencing

The cDNA was diluted to 0.2 ng/µl and a total of 1 ng was used for library preparation, performed as per the Nextera XT DNA Sample Preparation Kit protocol (Illumina). The resulting library was quantified and evaluated for fragment size using a 2200 TapeStation (Agilent Technologies), diluted to 10 pM and sequenced on an Illumina MiSeq platform (2 × 250 bp reads).

2.5. Data analysis

The MinION HDF5 files were processed using poretools version 0.6.0 (Loman and Quinlan, 2014) to extract read data in FASTA format. Adapters were removed from the MinION reads using Cutadapt version 1.9 (Martin, 2011). The trimmed MinION reads were aligned to the RRV strain QML1 genome reference sequence (GQ433354.1) using BWA-MEM version 0.7.7 (Li, 2013) with default parameters. The polyA tail was removed from the RRV reference sequence to improve reliability of the alignment. Coverage was calculated using SAMtools version 0.1.19 (Li et al., 2009), and similarity was calculated by determining the number of matches/mismatches at each base using pysamstats version 0.24.3 (available at: https://github.com/alimanfoo/pysamstats, accessed 10 January 2016). Variant calling was performed with BCFtools in the SAMtools package, with a quality threshold set at 200. The consensus sequence was derived and aligned to the RRV reference sequence using ClustalW in Geneious version 8.1.8 (Kearse et al., 2012). Taxonomic classification of the trimmed MinION reads was performed using a BLASTn version 2.3.0 search with the NCBI nucleotide database and the following parameters: e-value 1×10^{-5}; word size 28; and a maximum of one hit per read. Mosquito species identification was also performed using BLASTn with the same parameters and a custom database of *Cytochrome oxidase I* (COI) sequences from Australian mosquito species (Batovska et al., 2016).

The demultiplexed MiSeq reads were trimmed of adapters and bad quality bases using Trim Galore version 0.4.0 (Krueger, 2015). Reads were trimmed of bases with a PHRED quality score < 20 and were removed if they were < 100 bp long. In order to have comparable data, the trimmed MiSeq reads were normalised so that the total number of base pairs generated was similar to the total number of MinION base pairs. The normalised MiSeq reads were then analysed using the same method as for the MinION data.

The unprocessed MinION HDF5 files (SRR5572188) and MiSeq FASTQ files (SRR5572189) used in this study have been deposited into the National Centre for Biotechnology Information (NCBI) Sequence Read Archive under project ID PRJNA386415.

3. Results

3.1. Virus sequencing using the MinION

The viral load of the RRV-infected mosquito was estimated at 1.17×10^4 cDNA copies/µL using ddPCR.

When sequenced on the MinION, a total of 229 reads were identified as originating from RRV, accounting for 0.28% of the total reads

Table 1
Sequencing run data and Ross River virus (RRV) reference alignment statistics for the MiSeq and MinION. The mean read length is post-adapter trimming. The MiSeq raw reads are unpaired have been normalised according to total base pairs (bp).

	Raw reads	Total bp	Mean read length (bp)	Mapped reads to RRV (%)	Mean RRV coverage (x)	Pairwise identity (%)	% of ref. seq.
MinION	82,259	89,244,295	1,084.9	0.28	10	98.1	98.9
MiSeq	467,756	89,244,548	190.8	0.41	62	98.3	97.8

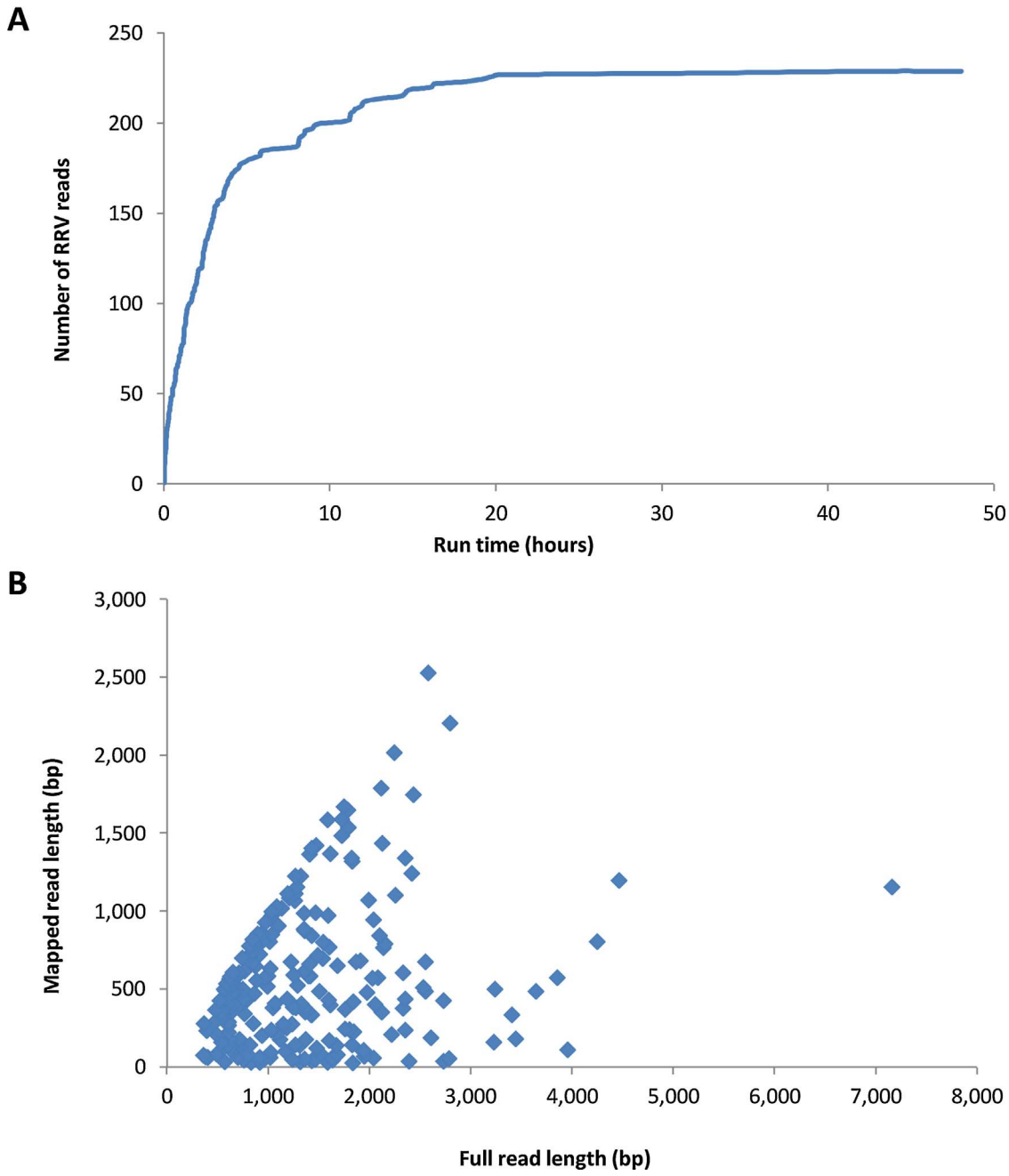

Fig. 1. Descriptive statistics of Ross River virus (RRV) reads generated by the MinION. A) The cumulative number of RRV reads generated throughout the 48 h sequencing run. B) A comparison of the length of the MinION reads that were mapped to the Ross River virus (RRV) reference sequence, compared to the original full length of the reads.

(Table 1). After 10 min of sequencing 12% of the RRV reads had been generated, increasing to 32.3% at one hour, and after 10 h the majority of RRV reads (87.3%) were sequenced (Fig. 1A). The generation of the RRV reads was proportional to the total number of reads generated during the course of the MinION run.

The MinION produced a range of RRV read lengths (mean: 1388.4 bp; range: 355–7159 bp); however, often large regions of the RRV reads produced by the MinION did not align to the reference sequence (Fig. 1B). A mean of 45.6% of the MinION read length aligned to the reference, with the remainder being clipped by the BWA-MEM aligner. Upon manual inspection, it was found that the clipped regions had higher levels of sequencing error compared to the mapped regions. After clipping, the longest RRV read length was 2528 bp and the mean RRV read length was 547.8 bp.

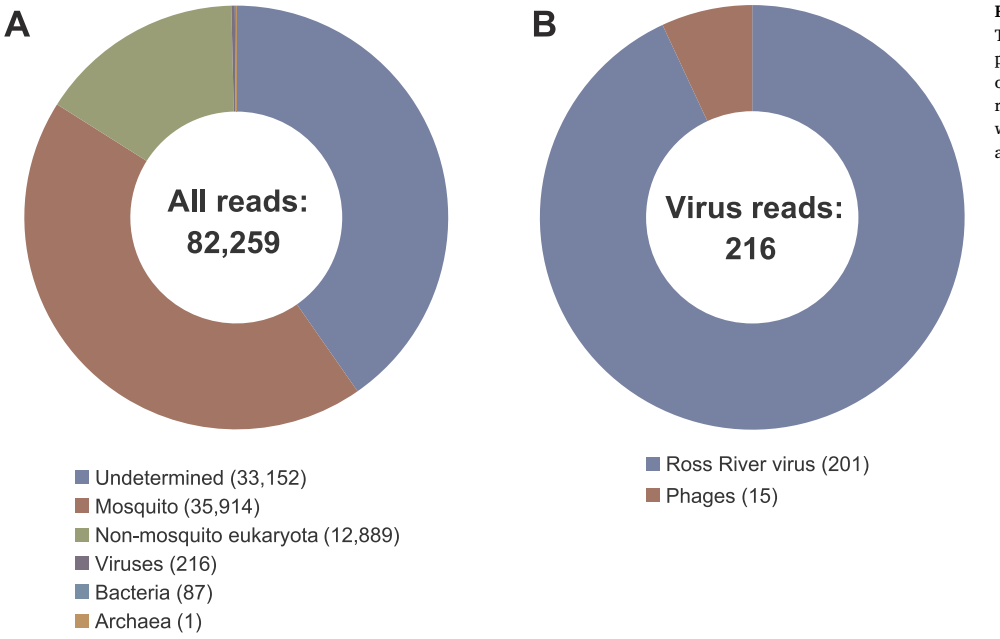

Fig. 2. BLASTn analysis of the MinION reads. A) Taxonomic classification of all the MinION reads, post adapter trimming. B) Taxonomic classification of the reads identified as virus. The total number of reads used is shown in the centre of the donut, whereas the number of reads assigned to each taxon are shown in brackets below the donut.

3.2. Taxonomic classification of the MinION reads

A BLASTn search using the NCBI nucleotide database allowed for characterisation of the MinION reads and untargeted detection of RRV. The majority of reads (59.3%) were classified as eukaryota, with most of these belonging to mosquito (Fig. 2A). Of the non-mosquito eukaryota reads, 79.9% were assigned to nematodes. The finding of nematode sequences was supported by the Illumina data, with 95.5% of non-mosquito eukaryote MiSeq reads belonging to nematodes. The majority of these reads belonged to a 4.2 kB region of the *Brugia timori* assembly (GenBank accession LK907835.1; 87.6% and 72.1% of MinION and MiSeq reads respectively). Of the 216 MinION reads that were identified as virus, 93.1% were RRV, with the rest classified as phage-related (Fig. 2B).

In addition to virus detection, the species of the host mosquito was successfully derived from the MinION data, with 100% of the mosquito COI BLASTn results matching *Ae. notoscriptus* (total = 24 reads).

3.3. MinION vs. MiSeq

The MinION generated poorer quality RRV reads than the MiSeq, with a 4.7% error rate compared to 0.7% for the MiSeq (Fig. 3A). The MinION also generated less RRV reads than the MiSeq (229 vs. 1919 reads), giving it less coverage of the genome (10-fold vs. 62-fold; Fig. 3B). However, the MinION RRV reads were longer than those produced by the MiSeq, with a mean RRV read length of 547.8 bp (range: 30–2528 bp) compared to 187.6 bp (range: 30–250 bp).

When comparing consensus RRV genome sequences, the performance of the MinION and MiSeq were comparable, with both platforms acquiring a similar percentage of the reference genome (98.9 vs. 97.8%), and similar pairwise identity (98.1 vs. 98.3%; Table 1).

Variant calling revealed a homozygous single nucleotide polymorphism (SNP) from the RRV reference genome at 10,065 bp. The SNP was present in both the MinION and MiSeq data (Fig. 3A).

4. Discussion

This study demonstrates the ability of the MinION to detect and sequence an arbovirus directly from an infected mosquito. The full-length RRV genome was recovered from the MinION reads, and a BLASTn search revealed the presence of RRV without targeting known viruses (Fig. 2). The BLASTn search also detected the presence of other organisms such as bacteria and nematodes, highlighting the MinION's potential to detect all the pathogens present within a mosquito. Due to the limited coverage of the nematode genome, the source of the nematode sequences requires further investigation. It should be noted that pathogen detection using metagenomic approaches is limited by the sequence databases available for classification.

The MinION is able to perform pathogen detection rapidly, with 12% of the RRV reads produced within the first 10 min of the run (Fig. 1A). Due to the MinION's real-time sequencing, reads can be analysed as they are generated, significantly speeding up virus detection (Walter et al., 2016). The ability to rapidly detect pathogens directly from a mosquito combined with the MinION's portability and affordability make it ideal for in-field biosecurity surveillance.

The utility of the MinION in biosecurity is further demonstrated by its ability to determine the species of the mosquito, in addition to detecting viruses. Despite being DNase-treated, reads specific to *Ae. notoscriptus* were present in the MinION data, leading to the correct identification of the mosquito host. Invasive mosquitoes such as *Ae. albopictus* are intercepted at international ports throughout the year, making species identification a vital aspect of biosecurity surveillance (Vaux and Medlock, 2015). Current methods involve morphological identification, which can be unreliable and difficult to perform on immature specimens. Molecular identification of species using a portable sequencer would allow decisions about vector control to be made quickly and help prevent the establishment of exotic mosquitoes.

This study supports previous findings (Greninger et al., 2015; Wang et al., 2015) that indicate virus characterisation using the MinION is comparable to that of the MiSeq. While the MinION had a higher error rate and lower coverage (Fig. 3), the pairwise identity and length of the two RRV consensus genomes was similar (98.1 vs. 98.3%; Table 1). Furthermore, even with a high error rate, the raw MinION reads could still be used for accurate virus detection (Fig. 2). The longer reads produced by nanopore sequencing facilitate the ability to accurately identify viruses, with reads as long as 2.5 kb mapping to the RRV genome. However, many of the mapped RRV MinION reads had regions clipped by the BWA-MEM aligner due to high error rate (Fig. 1B). Clipping could be reduced by error correcting the reads first with programs such as Nanocorrect (Loman et al., 2015). Alternatively, an aligner that does not clip reads could be used to analyse the data, although this could lead to decreased pairwise identity. Nevertheless,

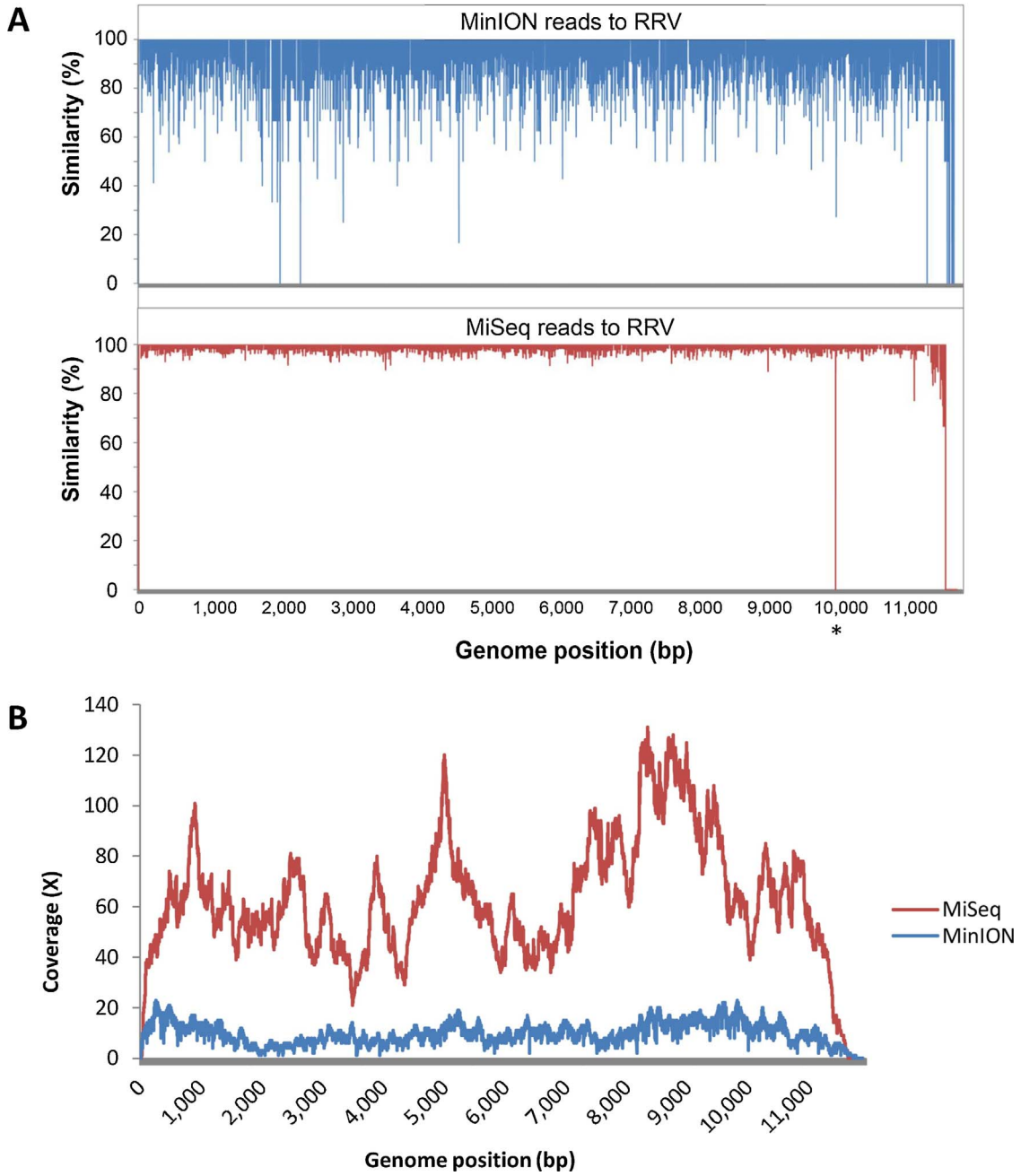

Fig. 3. Comparison of the A) similarity and B) coverage of MinION and MiSeq reads when aligned to the Ross River virus (RRV) reference sequence. The * symbol in **A** indicates the position of a single nucleotide polymorphism (SNP).

using BWA-MEM with uncorrected MinION reads remains sufficient for virus identification and directly comparable with short-read sequencing technology.

Now that the capacity of the MinION to sequence virus from a mosquito sample has been established, future studies can test the method in-field. A range of protocols and equipment have been designed to mobilise laboratory procedures and shorten turnaround time. For instance, the VolTRAX library preparation system, and the prospective SmidgION (Lu et al., 2016). However, the quality of the data produced by these devices has yet to be established. Improvements in bioinformatic processing also assist with in-field use, with the recent release of local basecallers, such as Nanocall (David et al., 2017), removing the need for an internet connection. Currently, the primary restriction to local basecalling is the large amount of computing power required; however, this will likely improve with time (Walter et al., 2016).

In-field use of the MinION would be further enabled with the Rapid 1D Sequencing Kit, which reduces library preparation time to approximately 10 min (Mitsuhashi et al., 2017). The results of this study indicate 1D reads are of sufficient quality for virus detection in mosquitoes. The direct sequencing of RNA would also reduce sample processing time, and has recently been demonstrated with the MinION (Garalde et al., 2016). The method currently applies only to polyadenylated RNA, but future advancements could expand this capability to all RNA and help avoid the biases associated with cDNA synthesis.

This study serves as a proof of concept for the metagenomic detection of an arbovirus from a mosquito sample using a MinION sequencer. This method of pathogen detection and characterisation differs from previous nanopore sequencing studies in that it uses the mosquito as the sample rather than clinical samples or viral cultures,

thereby providing a rapid and unbiased surveillance tool. Furthermore, no target enrichment is used, allowing virus detection without a priori knowledge. The MinION is affordable, portable, and able to detect arbovirus quickly and with sufficient accuracy, demonstrating its potential for in-field virus surveillance. Utilisation of recent protocols and device releases could allow virus detection on the MinION to be performed within a few hours in-field. Nanopore sequencing technology is advancing at an unprecedented pace, promising a future where portable sequencing will be routine in surveillance and many other fields.

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