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ORIGINAL ARTICLE

Adaptation of custom capture sequencing panels to the Oxford Nanopore MinION platform

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Abstract

Background Next generation sequencing (NGS) remains underutilized in clinical microbiology applications despite providing broad pathogen spectrum detection superior to other molecular methods. This is primarily because of lower sensitivity of metagenomic NGS (mNGS) compared to PCR, lengthy turn-around times, cost, and complexity of data analysis. Capture sequencing is a technique that can mitigate some of the limitations of mNGS. Using probes that are engineered to selectively bind and pull down desired nucleic acids, capture sequencing enriches for targets of interest and can result in up to a 10,000-fold increase in sensitivity compared to mNGS. In this study, we describe the application of capture sequencing on Oxford Nanopore Technology's portable sequencer, the MinION MK1C.

Methods We examined the performance of VirCapSeq-VERT and TBDCapSeq, two distinct capture sequencing assays that target vertebrate viruses and tick-borne pathogens, respectively. Both assays were originally established on the Illumina platform. To enable sequencing on the MinION instrument, we developed a modified hybrid workflow using our established library preparation and capture protocol for Illumina, followed by the addition of the ONT sequencing adaptor. In tests using contrived and clinical samples, we compared sensitivity thresholds and sequencing output, including pathogen genome coverage and relevant read counts.

Results The addition of capture enrichment to MinION NGS provided significant improvement in pathogen detection when compared to mNGS. Assessment of assay performance on pathogen-positive samples revealed equivalent sensitivity on the MinION MK1C and Illumina NextSeq. We found that the elevated read counts and sequencing depth generated by Illumina NGS were offset by the greater read length obtained on the MinION MK1C and resulted in comparable pathogen genome coverage between the two platforms.

Conclusion This study demonstrates the utility for employment of VirCapSeq and TBDCapSeq on different sequencing platforms and suggest the potential of the MinION platform for broad-spectrum clinical diagnostics.

Keywords Next generation sequencing · Minion · Illumina · Capture sequencing · VirCapSeq · TBDCapSeq

Abbreviations

CLEP	Clinical Laboratory Evaluation Program
LoD	Limit of detection
NGS	Next generation sequencing
ONT	Oxford Nanopore Technologies
qPCR	Quantitative polymerase chain reaction
TBDCapSeq	Tick-borne diseases capture sequencing

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Background

Over the past two decades, a wide range of technological innovations have spurred novel approaches for improving pathogen detection. During this time, PCR has become the gold standard rapid first line tool for diagnosis of infectious diseases, primarily because of its high sensitivity, speed, low cost, and assay simplicity. However, PCR is applicable only for detection of known or genetically related pathogens and has limited capacity for target multiplexing. The introduction of next generation sequencing (NGS) platforms has revolutionized research in infectious diseases by enabling pathogen surveillance, phylogenetics, and novel pathogen discovery [1–6]. Nonetheless, NGS has not yet gained wide acceptance in clinical microbiology likely due to limited sensitivity, operational costs, lengthy turn-around-times, and the computing power required for analyses of large data sets. Assay sensitivity of mNGS is also inferior to gold standard PCR tests [7–9]. Therefore, improvement in performance along with lowering sequencing costs are crucial for adopting NGS platforms in clinical microbiology.

To address the limitations of mNGS, we introduced a capture enrichment method that enhances assay sensitivity and reduces the complexity of bioinformatic analysis. The enabling component is a library of agent-specific biotinylated DNA probes that are designed along the entire length of a pathogen genome. The probes selectively bind and enrich the desired template prior to sequencing which subsequently results in a substantial reduction in background noise, simplifying bioinformatic analyses. The library is designed to include probes for a wide array of pathogens thereby enabling the broad range detection of unbiased NGS at sensitivities that can surpass PCR. We first described this approach in 2015 for pan viral detection (VirCapSeq-VERT) [7]. This was followed by an assay targeting all human pathogenic bacteria and their associated anti-microbial resistance (AMR) elements (BacCapSeq), and a targeted panel for pathogens associated with tick-borne infections (TBDCapSeq) [7, 9–11]. We validated VirCapSeq-VERT and secured regulatory Clinical Laboratory Evaluation Program (CLEP) certification from New York State Department of Health (NYSDOH) for use of this assay as a diagnostic test for multiple specimen matrices [8]. The advent of portable sequencers like the Oxford Nanopore Technologies (ONT) MinION provides an opportunity to move NGS from specialized laboratories to less well-resourced environments [12–14]. These portable devices and their current chemistry allow for rapid sequencing at a fraction of the cost of larger sequencers and the applicability of capture sequencing on the ONT platform has been shown [15–17]. Here, we report the adaptation of our current viral (VirCapSeq-VERT) and tick-borne agent capture sequencing (TBDCapSeq) probe sets to the ONT platform. This feasibility study demonstrates the potential diagnostic utility of this approach.

Materials and methods

ONT-VirCapSeq-VERT feasibility using ONT library preparation kits

Initial MinION feasibility tests were done using the cDNA Barcoding Kit (SQKPCB109) which employed oligo-dT primers for cDNA synthesis. For compatibility with the ONT kit components and workflow, we ensured that the sample input included polyadenylated mRNA transcripts, achieved by harvesting adenovirus-infected Vero cells to specifically target poly-adenylated viral mRNA transcripts. Total nucleic acids (TNA) from the infected cells were extracted using the NucliSENS EasyMag platform (bioMèrieux, France) and libraries were prepared as per the ONT kit protocol. Multiple parameters were subsequently evaluated, including sequencer run times, input concentrations, and library amplification PCR cycles, but we did not achieve an increase in sensitivity comparable to Illumina-VirCapSeq-VERT.

ONT-VirCapSeq-VERT feasibility using a custom workflow

Subsequently, we adopted an alternative approach and established a working protocol that used library preparation and capture kits from Twist Bioscience in conjunction with the ONT Ligation Sequencing Kit V14 (SQK-LSK114) and a sequencer run time of 15 h. This protocol was a modification of our current Illumina workflows wherein the capture enriched libraries are adapted to enable ONT sequencing. These alterations included shorter DNA fragmentation time to retain longer molecules, and the ligation of ONT sequencing adapters (SQK-LSK114) to the capture-enriched library pool to enable loading and sequencing on the MinION MK1C. The fragmentation step could not be omitted due to the kit configuration where the same enzyme mix (Twist Frag/AT Enzymes) carries out fragmentation, end repairs and A-tailing in one reaction. Thus the incubation was shortened to the minimal amount of time for the heat activation of the enzyme mix. This resulted in the fragmentation incubation decreasing from 5 min to 1 min, in order to retain longer DNA fragments.

The hybrid approach is illustrated in Fig. 1. This protocol employs random primers for cDNA synthesis, and we were no longer restricted to poly-adenylated RNA for input. Therefore, template material for these tests consisted of

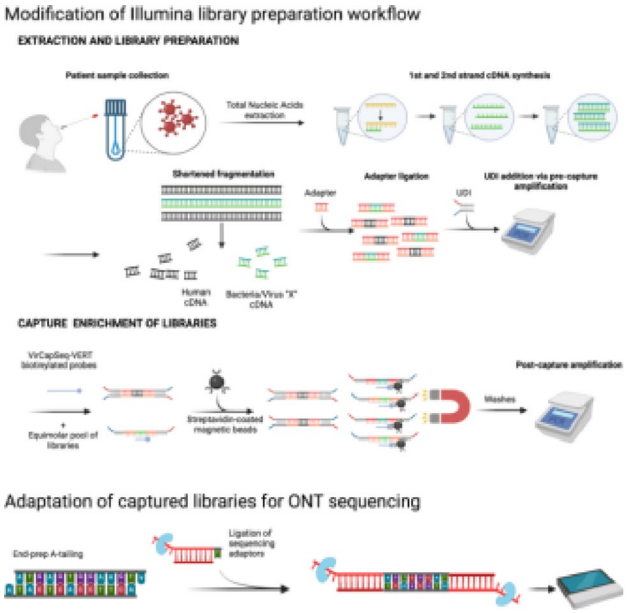

Fig. 1 Hybrid workflow for VirCapSeq-VERT on ONT platform. Illustration created in bioRender (Toronto, Canada)

TNA extracted on the EasyMag from samples comprised of quantified viruses spiked into a negative background matrix of nasal swabs. All subsequent testing for ONT-VirCapSeq-VERT were done on these contrived samples. After TNA extraction, first and second strand cDNAs were synthesized using random hexamers, Superscript IV (Random Hexamers and Superscript IV, Invitrogen, USA) and Klenow (Klenow Fragment (3′→5′ exo–) New England Biolabs, USA) followed by bead clean up (AMPure beads, Agencourt Axyprep, USA) and quality control (QC) checks on a Qubit instrument (Qubit, Thermo Fisher, USA). 50 ng of double-stranded cDNA (ds-cDNA) was used as input for preparing sequencing libraries. ds-cDNAs were subjected to fragmentation, end repair and dA-tailing followed by ligation of universal adapters using the Twist EF Library Prep 2.0 workflow (Twist Bioscience, CA, USA). Unique dual indices (UDIs) were introduced via library amplification. Indexed libraries were purified (AMPure beads, Agencourt Axyprep, USA) and quality-assessed using a TapeStation 4200 system (Agilent Technologies, CA, USA). Equal amounts (by mass) of the uniquely indexed libraries were pooled to obtain a total DNA mass of 1,500 ng. The pool was capture-enriched using our custom VCS probes and Twist Fast Hybridization Kit (Twist Biosciences, USA), and was recovered using streptavidin-coated magnetic beads, washed under high-stringency conditions as per the Twist SOP, and amplified by post-capture PCR (13 cycles). The enriched pool was purified, quantified using the TapeStation 4200, and ligated to ONT sequencing adapters using the Ligation Sequencing Kit V14 (SQK-LSK114; Oxford Nanopore Technologies, UK).

Performance comparison of ONT-VirCapSeq-VERT over ONT-mNGS

A random assortment of six viruses spiked in at varying concentrations were selected for analysis of *ONT-VirCapSeq-VERT* performance. Individual viral libraries with discrete barcodes were pooled, captured and loaded on a MinION MK1C. The same pooled libraries were also sequenced in parallel without capture, using mNGS. All viruses were assayed in duplicate runs with both ONT-mNGS and ONT-VirCapSeq-VERT.

Sensitivity comparison of Illumina-VirCapSeq-VERT and ONT-VirCapSeq-VERT

For assessing linearity and the limits of detection (LoD) on Illumina, viruses were quantified in copies/mL using virus-specific qPCR assays and then spiked in to the background matrix at concentrations starting from 500,000 down to 0.5 copies/mL in 10-fold serial dilutions. As part of VCS feasibility testing on the ONT we wanted to investigate if VirCapSeq-ONT could detect all viruses at the Illumina based LoDs. Thus, we conducted runs only at those concentrations by spiking in each virus at its previously established Illumina threshold [8]. Six discretely barcoded libraries representing viruses at their Illumina-based limit of detection (LoD) and sensitivity thresholds were included in each run and compared across two independent runs for each virus.

TBDCapSeq pathogen quantification and sample preparation

Generation of quantitative PCR assays

Individual Taqman-based quantitative PCR (qPCR) assays were designed for *Anaplasma phagocytophilum*, *Babesia microti*, and *Borrelia burgdorferi* (Supplemental Table 1). A positive control standard was obtained from GenScript, linearized, quantified, and serially diluted from 10^9 to 10^0 copies/µl in background of salmon sperm DNA (5ng/µl). QPCR assays were performed with AmpliTaq gold 360 DNA polymerase (ThermoFisher Scientific) using 2 µl of template DNA.

Clinical and contrived samples for TBDCapSeq

Both clinical and contrived whole blood samples were used for ONT-TBDCapSeq testing. Clinical samples were obtained from the Lyme biobank [11, 18]. All were previously comprehensively tested at clinical laboratories for infection/exposure to multiple tick-borne pathogens using serology and/or PCR. DNA was extracted directly from 200

µl of whole blood using QIAamp DNA Blood Mini Kit (Qiagen, MD, USA). To generate contrived specimens, aliquots from human whole blood were obtained from the Department of Pathology at Columbia University. Clinical whole blood samples with high pathogen burden for *B. microti* and *(A) phagocytophilum* were used as a source for subsequent spiking experiments. For *(B) burgdorferi*, live cultured spirochetes from a low passage, infectious B31 strain were added directly to whole blood. For *B. microti* and *(A) phagocytophilum*, clinical whole blood samples were extracted, quantified and then approximately 10^5 genomic copies were spiked and serially diluted in whole blood-derived DNA. For *(B) burgdorferi*, the serial dilutions were done in culture media, quantified, and then live spirochetes were added to whole blood and extracted. Samples with pathogen concentration between 1 and 3 genomic copies per µl, as determined by qPCR, were selected as targets for reproducibility assays. To measure precision, 9 unique replicates of each target pathogen were processed. Additionally, DNA extracted from a negative whole blood sample was tested to characterize the background and ensure that no signals were detected for pathogens targeted by the TBDCapSeq assay. Samples were analyzed under Columbia IRB protocol AAAT5727.

ONT-TBDCapSeq library preparation

DNA concentration was measured with the Qubit High Sensitivity Double-stranded DNA kit and Qubit 2.0 Fluorometer (Invitrogen, MA, USA). Sequencing libraries were prepared with the KAPA HyperPrep kit (Roche, MA, USA) using 100 ng of DNA. Universal adapters were ligated to the DNA and then amplified using Dual Index (DI) Primer Mixes (Roche, MA, USA). Amplified libraries were quantified using the TapeStation 4200 (Agilent Technologies, CA, USA). After quantification, equal amounts (by mass) of each uniquely indexed library were pooled (ranging from 3 to 4) to obtain a combined DNA mass of 1,500 ng. We employed Roche-manufactured capture probes, as these were the probes used for all previously published TBDCapSeq experiments [9, 11]. Following an overnight incubation at 55 °C (16–20 h), the captured libraries were pulled down using magnetic streptavidin beads (KAPA HyperCapture Bead kit) and washed. The enriched library pool was subjected to post-capture amplification and quantified on the Tapestation 4200. ONT sequencing adaptors were ligated to the enriched library pool using the Ligation Sequencing Kit V14 (SQK-LSK114, Oxford Nanopore Technologies, UK).

ONT Sequencing

As per ONT SOP, 200 fmol of the DNA library pool was subjected to end-repair and dA-tailing followed by a purification with AMPure XP Beads (Agencourt Axyprep, USA) at a 1:1 ratio. Adaptors were ligated and following a bead-based purification, the final library was quantified using the Tapestation 4200 and diluted to 20 fmol. Libraries were loaded onto primed R10.4.1 flow cells (FLO-MIN114). Sequencing was carried out on a MinION MK1C.

Bioinformatics

Raw Nanopore FASTQ files were demultiplexed using Oxford Nanopore Guppy barcoder v. 6.5.7 + ca6d6af and quality filtered using Fastp v 0.23.4 (minimum read length: 400 bp; minimum quality score: 10 [19, 20]. Host subtraction was performed using the human genome (GRCh38) with Minimap2 v 2.28-r1209, and unmapped sequences were extracted using Samtools 1.20 [21, 22]. Non-host reads were mapped to a reference database of pathogen genomes using Minimap2 [22], retaining only reads with mapping quality scores > 10. A BLAST analysis (Blastn, megablast) against a curated pathogen database was performed, extracting the optimal alignment per read [23]. For VirCapSeq, the database consists of all vertebrate viral sequences while the tick-borne pathogen database includes microbial genomes associated with tick-borne disease. Further filtering was applied using a Python v. 3.12.2 script to refine pathogen assignments [24]. CIGAR string processing was conducted to retain high-confidence alignments, and supplementary alignments were removed. Final filtered reads were converted to BAM format, sorted, and indexed using Samtools [21]. Read coverage and mapping statistics were generated with Bedtools v 2.26.0 and Samtools idxstats [21, 25], and a summary report was compiled using a custom Python script. Positivity thresholds were established based on analyses of control samples using a custom bioinformatics pipeline. For Illumina, final host-subtracted reads were subjected to homology search using GenBank MegaBLAST against the curated custom viral database. All reads were subsequently re-mapped to the reference viral sequences identified by the initial homology search to validate the MegaBLAST results. Finally, stringency filters including e-value of $\leq$ 1.00E-35, nucleotide identity of $\geq$ 95%, alignment lengths of $\geq$ 100nt, minimum number of unique reads aligned (N $\geq$ 25), minimum number of genomic regions $\geq$ 100nt (N $\geq$ 3), and cumulative minimum length of $\geq$ 500nt were applied. For ONT sequencing, because of lower fidelity, lower sequencing total read output and longer read length, the stringency filters were adjusted to include nucleotide identity of $\geq$ 85%, alignment lengths of $\geq$ 300nt, minimum number of genomic

regions ($N = 2$), and cumulative minimum genome coverage of 0.20%.

Results

ONT-VirCapSeq-VERT

Quantified adenovirus TNA was extracted from infected cells as template for all initial proof-of-concept assays. To determine a suitable sequencing run time using ONT standard protocols, results of five and 15-hr run times were compared, without the addition of VirCapSeq-VERT enrichment (Supplemental Table 2). The 15-hr run time proved optimal and was selected for subsequent assays that now incorporated VirCapSeq-VERT enrichment to the standard ONT's SOP. The kit chemistry did not allow for a post capture amplification step unless the same universal barcoding primers were used again, a suboptimal restriction that would likely promote non-specific amplification. Therefore, the assay was performed without the post capture PCR amplification that is part of our established Illumina-VirCapSeq-VERT SOP. The total raw and viral-specific reads were lower when compared to the ONT-mNGS run without capture, despite increasing the concentrations of input TNA (Supplemental Table 2). This was expected as the majority of host and environmental nucleic acids are depleted as part of the capture process that requires the post capture amplification step to augment the total mass of captured DNA. Accordingly, for subsequent runs the universal library amplification primers were used to include a post-capture amplification step. The sequencing libraries were divided into three equal portions, with one portion sequenced without capture (unbiased), one with capture enrichment but without post-capture PCR amplification and the last with a post-capture amplification step. Even the addition of a post-capture amplification step did not increase sensitivity comparable to Illumina instruments leading to a conclusion that the ONT standardized workflows for the kits used for feasibility testing were incompatible with our capture protocol. The assays were then run with a modified hybrid workflow using our established library preparation and capture protocol for Illumina-VirCapSeq-VERT followed by the addition of the ONT sequencing adaptor to enable sequencing on the MinION instrument. The fragmentation time of DNA was shortened, to enable long read sequencing. The modified workflow resulted in significant enrichment, when compared to mNGS. The comparisons of ONT-mNGS and ONT-VirCapSeq-VERT were done in duplicate using contrived samples. Both runs yielded comparable data and demonstrated that the addition of capture enrichment resulted in up to 6000-fold increase in sensitivity (Table 1). The capture

Table 1 Comparison of metagenomic NGS vs. VirCapSeq-VERT

Virus	Input Concentrations (copies/ml)	ONT-mNGS Normalized Raw Reads/M*	ONT-mNGS Normalized Viral Reads/M*	ONT-mNGS On Target Reads* (%)	ONT-VCS Normalized Raw Reads/M*	ONT-VCS Normalized Viral Reads/M*	ONT-VCS On Target Reads* (%)	Fold Enrichment*
Human adenovirus C	50,000	61,156	0	0.000	33,742	1,286	3.811	-
Human coronavirus OC43	50,000	81,438	95	0.117	71,237	39,050	54.817	~500
SARS-CoV-2	50,000	69,448	19	0.027	83,573	66,500	79.571	~3500
Enterovirus D68	50,000	82,198	31	0.038	74,006	33,600	45.402	~1000
Human metapneumovirus	50,000	72,988	79	0.108	47,280	31,459	66.537	~400
Respiratory syncytial virus A	50,000	65,917	117	0.177	101,534	68,910	68.547	~600
Influenza A	50,000	59,541	0	0.000	100,002	390	0.390	-
Human parainfluenza virus 1	50,000	83,512	47	0.056	94,387	44,100	46.722	~1000
Human coronavirus 229E	5,000	105,832	2	0.002	77,910	11,300	14.504	~6000
Human parainfluenza virus 2	5,000	70,934	0	0.000	117,180	1,352	1.154	-
Influenza B	50	34,383	0	0.000	23,991	3	0.013	-
Human parainfluenza virus 3	50	44,803	0	0.000	31,112	2	0.006	-

*Mean of two runs

ONT-mNGS: metagenomic sequencing on the MinION MK1C

ONT-VCS: VirCapSeq-VERT sequencing on th MinION MK1C

Table 2 Comparison of VirCapSeq-VERT sensitivity thresholds on Illumina vs. ONT

Virus	LoD concentrations (copies/ml) established on Illumina	Viral specific reads on Illumina	Viral genome coverage on Illumina	Viral specific reads on ONT*	Viral genome coverage on ONT*
Adenovirus C	50	3442	29.69%	134	21.69%
Enterovirus D68	5	2483	78.82%	432	90.28%
Influenza A virus	50	1356	37.84%	22	24.84%
Influenza B virus	50	2029	38.03%	49	34.91%
Respiratory syncytial virus	5	1177	81.82%	785	89.19%
Human metapneumovirus	50	1653	40.70%	47	31.27%
Human parainfluenza virus 1	5	2012	60.03%	333	99.35%
Human parainfluenza virus 2	5	1995	98.43%	124	72.21%
Human parainfluenza virus 3	50	4373	48.32%	48	38.05%
Coronavirus 229E	50	5118	85.85%	220	95.00%
Coronavirus OC43	50	984	32.04%	64	55.38%
SARS-CoV-2	50	2323	47.13%	177	67.37%

*Mean of two runs

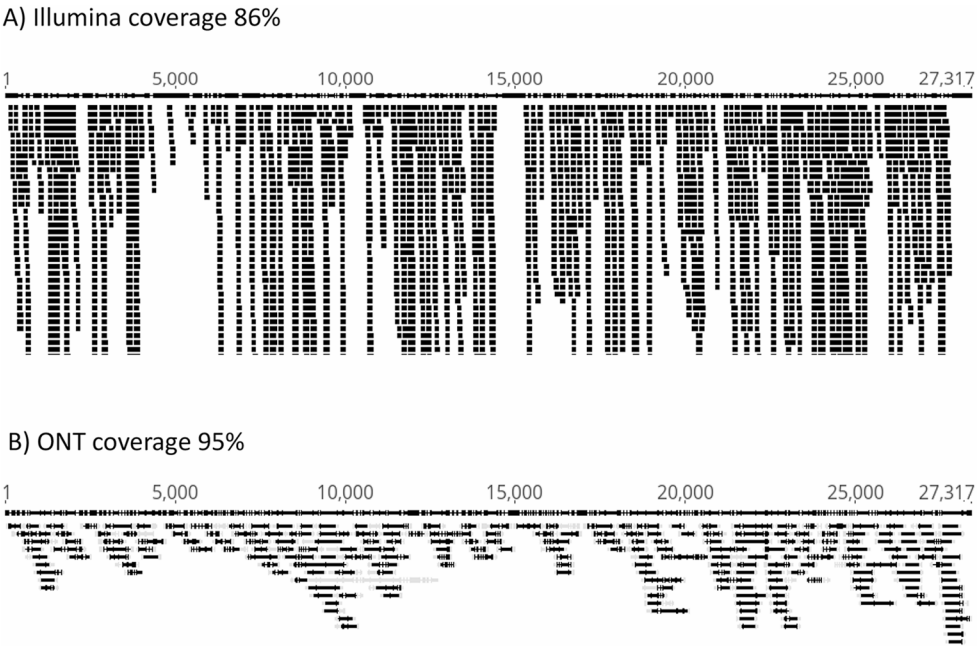

Fig. 2 Comparison of VirCapSeq-VERT results on the Illumina (panel A) and ONT (panel B) platforms. Shown are the sequencing reads mapped to for human coronavirus 229E (accession number NC_002645.1; www.ncbi.nih.gov; accessed 06/10/2025) sequenced at its sensitivity threshold (50 copies/ml) on both platforms. The Illumina NextSeq 2000 150 bp single end sequencing with VirCapSeq-VERT yielded 5,118 229E specific reads with a genome coverage of 85.85%. VirCapSeq-VERT with ONT yielded 220 229E specific reads with maximum read length of 1.3KB, a mean read length of 800 bp, and a genome coverage of 95%

enrichment also enabled the detection of human parainfluenza virus 3 and influenza B at their Illumina established LoD concentration of only 50 copies/ml. Therefore, additional tests were performed for other viruses to determine if VirCapSeq-VERT sensitivity on the ONT was comparable to previously established VirCapSeq-VERT thresholds on the Illumina platform [8]. The ONT-VirCapSeq-VERT results across the two runs matched closely in terms of viral specific reads and viral genome coverages, and the sensitivity was similar and reproducible on both platforms (Table 2).

We previously demonstrated that capture enrichment in conjunction with Illumina's sequencing by synthesis (SBS) chemistry for short fragments enabled between a 1,000–10,000-fold gain in sensitivity and higher genome coverage when compared to mNGS [7]. We hypothesized that despite a significantly lower number of output reads, the long chain sequencing on Nanopore technologies may allow for a comparable genome coverage with capture enrichment due to the longer read lengths. Our results confirmed that genome coverage was similar for both platforms (Fig. 2).

ONT-TBDCapSeq

Next, we tested the adaptability and performance of TBDCapSeq on the MinION MK1C. First, we needed to confirm that the addition of the capture step would enhance sensitivity relative to mNGS, similar to what we observed

with Illumina sequencing and for ONT-VirCapSeq-VERT. Three whole blood clinical samples were selected for testing, each previously found to be positive for a different tick-borne agent (*Borrelia burgdorferi*, *Babesia microti*, and *Ehrlichia chaffeensis*) with qPCR and/or Illumina NextSeq 2000-based TBDCapSeq (Table 3). Notably, the pathogen burden in the *B. burgdorferi* sample was low, testing negative by our in-house qPCR and by an outside clinical testing site [18]. Individual sequencing libraries were generated for each sample and then pooled. Two identical pools were analyzed separately on the MinION MK1C, one using a mNGS approach, and the other pool using capture sequencing.

Pathogen detection using ONT-mNGS was poor (Table 3). *Borrelia burgdorferi* and *E. chaffeensis* were not detected and only 3 sequencing reads were obtained for *B. microti*, the agent with the highest pathogen burden in the pool. However, a substantial improvement was achieved in the quantity of agent specific sequencing reads with ONT-TBDCapSeq (Table 3). All three pathogens were detected, including *B. burgdorferi* within the qPCR-negative sample.

Next, contrived samples were tested, wherein *B. burgdorferi*, *B. microti* and *(A) phagocytophilum* were spiked into whole blood to determine assay precision and detection thresholds for ONT-TBDCapSeq. For each agent, nine individual samples were prepared, all at similar pathogen concentration, as determined by qPCR. Each blood sample was extracted, and an individual sequencing library was prepared. A total of twenty-seven libraries were prepared, nine from each agent. For NGS sequencing, pools were processed, each consisting of three sequencing libraries, one from each pathogen. The mean pathogen concentrations for the nine pools were determined by qPCR to be 1–2 genome equivalents (GE)/µl for each agent and up to 2 µl was used for each library. All three agents were detected in every pool, although the number of reads and genome coverage was higher for *(B) microti* and *(A) phagocytophilum* despite similar template input (<4 GE per agent) (Table 5). Nonetheless, a high degree of assay reproducibility was achieved and sensitivity thresholds for *(B) burgdorferi*, *B. microti* and *A. phagocytophilum* were established as being under 4 GE per assay. A library generated from a healthy control did not produce sequencing reads for any of the three agents.

To evaluate the performance of ONT-TBDCapSeq relative to Illumina, additional whole blood specimens were examined, all previously tested with TBDCapSeq on the Illumina NextSeq 2000. Seven samples with low pathogen read count were selected, including three that were qPCR-negative (Table 5). Two other samples were obtained from healthy controls who were negative for all pathogens tested. Individual sequencing libraries were combined into pools of three prior to capture and MinION MK1C sequencing. All pathogen-positive samples were identified accurately

Table 3 Comparison of TBDCapSeq and metagenomic NGS

Sample ID	Clinical diagnosis*	TBD Agent	qPCR Ct	TBDCapSeq-ONT			Metagenomic NGS		
				Mapped Reads	Normalized reads (pathogen reads per million total reads)	% Chromosome Coverage	Mapped Reads	Normalized reads (pathogen reads per million total reads)	% Chromosome Coverage
LYM-2007	*Lyme disease - serology*	*Borrelia burgdorferi*	Negative	153	90.8	0.6	0	0	0
LYM-2002	*Ehrlichiosis -PCR*	*Ehrlichia chaffeensis*	32	1,558	1111.7	6.6	0	0	0
LYM-1985	*Babesiosis-PCR*	*Babesia microti*	27	259,254	144,557.7	91.6*	3	9.5	0.05*

* Diagnoses were made by reference clinical laboratories.

** Includes the mean coverage of the four *B. microti* chromosomes.

ND; Not done

Table 4 TBDCapSeq assay precision on the MinION MK1C

Replicate	Raw reads	TBD Agent	ONT-TBDCapSeq		qPCR	
			Mapped reads	% Genome coverage**	Ct	Copies/µl
		B. burgdorferi	190	2.6	40.0	0.5
Pool 1	8,865,159	*B. microti*	1,713	1.1	40.1	0.5
		A. phagocytophilum	1,793	7.4	38.8	1.5
		B. burgdorferi	179	2.7	39.8	0.6
Pool 2	9,115,364	*B. microti*	2,138	3.7	40.1	0.6
		A. phagocytophilum	2,888	17.2	39.3	1.1
		B. burgdorferi	14	0.2	40.0	0.5
Pool 3	9,978,686	*B. microti*	1,483	1.8	40.0	0.6
		A. phagocytophilum	7,021	36.8	39.3	1.1
		B. burgdorferi	71	1.15	NA*	-
Pool 4	9,414,377	*B. microti*	2,252	3.8	40.9	0.3
		A. phagocytophilum	7,092	45.3	38.4	2.1
		B. burgdorferi	40	0.6	39.8	0.7
Pool 5	8,419,029	*B. microti*	1,227	1.5	39.2	1.2
		A. phagocytophilum	2,335	19.1	38.3	1.9
		B. burgdorferi	202	2.25	39.7	0.7
Pool 6	10,847,499	*B. microti*	1,927	1.8	40.4	0.5
		A. phagocytophilum	3,104	12.6	38.4	1.9
		B. burgdorferi	45	0.25	40.0	0.5
Pool 7	10,060,059	*B. microti*	1,381	1.8	38.7	1.5
		A. phagocytophilum	3,991	15.5	39.2	1.1
		B. burgdorferi	134	1.62	39.8	0.6
Pool 8	7,733,645	*B. microti*	945	1.3	40.2	0.5
		A. phagocytophilum	3,364	19.2	39.1	1.3
		B. burgdorferi	93	0.8	39.8	0.7
Pool 9	9,967,215	*B. microti*	1,431	2.0	40.1	0.6
		A. phagocytophilum	2,911	13.0	38.8	1.9
		B. burgdorferi	0	0	*	0
Healthy control^	6,241,599	*B. microti*	0	0	*	0
		A. phagocytophilum	0	0	*	0

Table 5 Analysis of clinical samples with TBDCapSeq-ONT

ONT Pool #	Sample ID	Agent detected	Illumina TBDCapSeq**			TBDCapSeq-ONT		
		by TBDCapSeq	Agent reads	Filtered reads	Reads/million	Agent reads	Filtered reads	Reads/ million
Pool 1	LYM-1385	Borrelia miyamotoi*	608	8310969	73.2	138	618019	222.6
	LYM-1308	Rickettsia rickettsii^	465	12105761	38.4	121	566221	212.3
	LYM-2127	Ehrlichia chaffeensis^	218	3359795	64.9	117	570653	205.3
Pool 2	LYM-1999	Ehrlichia ewingii^	1289	19333240	66.8	18	403796	45
	LYM-2038	Borrelia burgdorferi*	307	16181451	19	38	284934	135.7
	LYM-2040	Healthy control	0	8981212	0	0	275668	0
Pool 3	LYM-1983	Borrelia burgdorferi*	168	10619998	15.8	13	795946	16.5
	LYM-1442	Anaplasma phagocytophilum	854	1489500	573.2	9646	2291569	4212.2
	LYM-2041	Healthy control	0	13421785	0	0	881384	0

*agent-specific PCR negative

^PCR was not performed

**samples were sequenced in pools 20–30

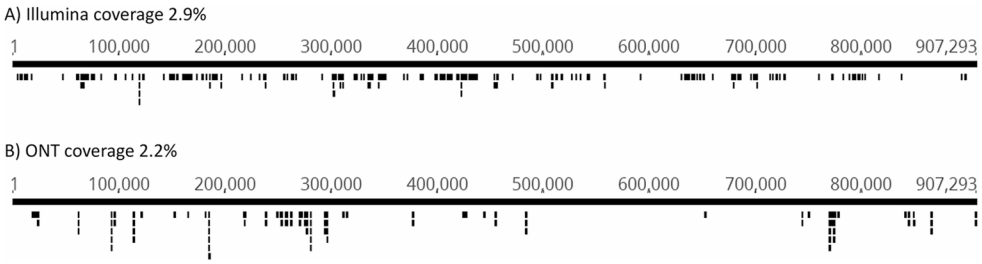

Fig. 3 Genome coverage comparison of TBDCapSeq using Illumina (panel A) and ONT (panel B). Shown are the reads aligned to the chromosome of *B. miyamotoi* strain LB-2001 (accession number NC_022079.2; www.ncbi.nih.gov; accessed 06/17/2025) generated from a whole blood *B. miyamotoi*-positive clinical sample

and after normalization to reads per million, the read count and the genome coverage were comparable between the two platforms (Table 5; Fig. 3).

Discussion

Rapid differential diagnosis of infectious diseases can guide decision making crucial for public health including the introduction of appropriate containment measures. It also enables better patient management through the selection of interventions that reduce morbidity, mortality and healthcare costs. Because signs and symptoms of infectious diseases are rarely specific for individual pathogens, laboratory tests are critical for clinical insights. PCR assays have become mainstream in clinical microbiology. However, even multiplex PCR has limited capacity for targeting a wide repertoire of pathogens, may fail to detect novel pathogens, and do not provide detailed phylogenetic data. For example, the emerging SARS CoV-2 virus would not have been detected using existing PCR assays. However, VirCapSeq probes, designed in 2015, facilitated complete genome assembly of this virus [15, 16]. mNGS circumvents some of the limitations of PCR, but it is less sensitive. In contrast, capture sequencing provides the sensitivity of PCR and tolerates sequence divergence similar to mNGS. Unlike PCR, we target complete genomes for probe design, and the probes can tolerate up to 30% of nucleotide divergence. This strategy allows for detection of novel, highly divergent pathogens through probes that hybridize within conserved regions of the genome. While there could be a drop in genome coverage within the variable regions of emerging or novel strains, the assay would not fail to detect a novel agent. The enrichment through positive selection also lowers the sequencing depth that is required for mNGS and thereby facilitates the analysis of multiple samples within a single assay and substantially lowers per-sample costs. Typically, a single VirCapSeq-VERT assay on an Illumina NextSeq 2000 can provide pathogen genomic analyses for up to 50 samples. In our previously reported validation efforts, VirCapSeq-VERT displayed a high degree of concordance with the FDA approved BioFire Respiratory Panel and even identified co-infections missed by the initial testing [8]. Nonetheless, batching larger sample numbers can become a limitation in the clinical setting when a quick diagnosis for a single or a few critically ill patients is required. Here the ONT is ideally suited since it allows for single sample analysis at economic costs. Another benefit of the smaller ONT instruments is the portability for field deployment as part of an outbreak response.

Because of the advantages provided by ONT sequencing, we undertook this feasibility study to demonstrate the utility of our capture sequencing panels on the MinION instrument. The aim of the current study was not intended to directly compare the outputs of ONT and Illumina. Rather, we only sought to demonstrate the flexibility of our capture assays, irrespective of the sequencing platform used, and illustrate the potential advantages provided by ONT capture sequencing. We were able to successfully adapt our current hybrid protocol onto the MinION with the significant sensitivity enhancement that is typical of capture sequencing relative to mNGS. For VirCapSeq-VERT, we have previously established LoD thresholds on Illumina sequencers [8]. Here, we were able to match these thresholds on the MinION. As anticipated, the variant chemistries employed by Illumina and ONT produced stark differences in read output. In comparisons of Illumina and ONT, the number of pathogen-specific reads provided by Illumina sequencing was substantially greater and directly correlated with greater overall read counts. However, this disparity was mostly offset by the longer sequencing reads provided by the MinION and the genomic coverage between the two platforms was comparable. Thus, our results demonstrate the benefits of combining capture sequencing with ONT instruments.

Despite the utility of NGS for diagnostics, there are challenges that need to be addressed for this method to gain acceptance in clinical laboratories. The establishment of clear positivity thresholds is key for diagnostic utility. On Illumina, our positivity threshold is a combined function of the overall read number and sequence length generated,

number of discrete genomic regions detected, and % genome recovery. Implementing identical parameters on the ONT platform is impeded by lower read output and sequencing fidelity. We established an analysis that utilized homology search to curated pathogen databases using BLAST and a confirmation alignment to a reference genome. A sample is called positive if BLAST and mapping results correlate along with a minimum genome recovery of 5% for viruses and 0.2% for cellular pathogens from two distinct regions of the genome. Finally, to reduce turnaround time for ONT sequencing while increasing sensitivity and specificity, we will explore adaptive sampling that selectively sequences DNA molecules of interest and discards host or other designated nucleic acids [26, 27].

Conclusion

Technological advances have reduced the cost of NGS and enhanced assay performance. However, the infrastructure required to support NGS can render it cost-prohibitive and impractical for point-of-care testing. Additionally, as demonstrated with the analyses of the clinical samples from patients with tick-borne disease, the low sensitivity of mNGS remains one of its key weaknesses as a diagnostic tool. The inclusion of enrichment methods such as capture sequencing are critical for providing acceptable thresholds for detection. Our capture systems, VirCapSeq and TBDCapSeq, are highly adaptable, reproducible and robust as demonstrated with the use of different probes manufacturers and sequencing instruments. This suggests that investment into NGS-based diagnostics can provide economical, sensitive and specific tests with broad-pathogen coverage that can potentially be implemented in future point of care setting.

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Data availability Raw sequence reads originated from this study has been submitted in the NCBI Sequence Read Archive under BioProject ID SUB15617694 and is publicly available.

Declarations

Ethics approval and consent to participate NA.

Consent for publication NA.

Competing interests The authors declare no competing interests.

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