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Commentary

# An evolution of Nanopore next-generation sequencing technology: implications for medical microbiology and public health

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**ABSTRACT** Next-generation sequencing has evolved as a powerful tool, with applications that extend from diagnosis to public health surveillance and outbreak investigations. Short-read sequencing, using primarily Illumina chemistry, has been the prevailing approach. Single-molecule sensing and long-read sequencing using Oxford Nanopore Technologies (ONT) has witnessed a breakthrough in the evolution of the technology, performance, and applications in the past few years. In this issue of the *Journal of Clinical Microbiology*, Bogaerts et al. (https://doi.org/10.1128/jcm.01576-23) describe the utility of the latest ONT sequencing technology, the R10.4.1, in bacterial outbreak investigations. The authors demonstrate that ONT R10.4.1 technology can be comparable to Illumina sequencing for single-nucleotide polymorphism-based phylogeny. The authors emphasize that the reproducibility between ONT and Illumina technologies could facilitate collaborations among laboratories utilizing different sequencing platforms for outbreak investigations.

The transformation of DNA sequencing started with the advent of the massively parallel, short read, next-generation sequencing (NGS) over the first decade of the 21st century. Illumina-based sequencing emerged as the most widely adopted NGS technology worldwide, utilizing sequencing by synthesis. The applications of Illumina NGS included targeted and whole-genome sequencing, transcriptome analysis, metagenomics, among others (1). Illumina-based sequencing has solidified its position as the gold standard due to its high accuracy. However, short reads have presented challenges in assembling complete genomes, particularly in genomes containing repetitive regions. Alongside an urgent demand for rapid and cost-effective sequencing, there was a significant market opportunity for innovative solutions. Oxford Nanopore Technologies (ONT), founded on electronic, disruptive, single-molecule sensing and nanopore science, originated as an idea in 1989, was established in 2005 and has made substantial progress over the past decade (Table 1).

Professor David Deamer conceived the initial idea in June 1989: a protein channel could be integrated into a liposomal membrane, allowing nucleotides to pass through the channels, blocking ionic movement, and resulting in size-proportional changes in current. It is remarkable to observe Professor Deamer's handwritten sketch in his notebook from Sunday, 25 June 1989, where his envisioned graphic of current change bears a striking resemblance to the current raw data output of ONT sequencing (2). However, experiments did not commence until 1993 under the leadership of Harvard Professor Dan Branton, eventually leading to the filing of multiple patents (3). It was not until 2005 that Oxford Nanopore was founded, with initial efforts focused on developing nanopore sequencing chemistry and platforms capable of high-throughput measurement of picoamp currents. Subsequently, large arrays were swiftly developed, and in

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**TABLE 1** Illumina versus Nanopore-sequencing technologies

| | Illumina | Nanopore (ONT) |
|---|---|---|
| Sequencing technology | Clonal array formation and reversible terminator technology<br>Cluster generation<br>Sequencing by synthesis<br>Bridge amplification<br>Parallel and large scale | Single molecule<br>Monitoring electric current changes as DNA or RNA fragments of any length pass through a protein; nanopore |
| Advantages | Accuracy<br>Robust basecalling particularly for homopolymer and repetitive regions<br>Scalability and different flow cell designs | Long reads, unrestricted read length<br>Real-time analysis<br>Can sequence native DNA or RNA<br>Simplified library preparation solutions<br>Scalability and different flow cell designs<br>Adaptive sampling (targeted sequencing with no library enrichment steps) |
| Nucleic acid fragments | Short (maximum read length 2 × 300 bp) | From short to ultra-long |
| Sequencers | iSeq100<br>MiniSeq<br>MiSeq Series<br>NextSeq 550 Series<br>NextSeq 1000 & 2000<br>NovaSeq 6000 Series<br>NovaSeq X Series | MinION<br>MinION Mk1C<br>GridION<br>PromethION P2i/P2 Solo<br>PromethION P24/P48 |

2014, the MinION early access program was initiated, granting researchers access to early-stage MinION devices (4, 5).

In 2016, the R9 nanopore, offering enhanced performance, was released. Compared to the previous R7 flow cells, R9 significantly improved sequencing accuracy. The R9 technology employed a 1D method, reading only one strand of DNA, thereby simplifying workflow, enabling rapid sample preparation protocols, and facilitating the collection of much larger data sets (6). Coupled with advancements in basecalling algorithms and the generation of long reads, the R9 chemistry was widely used among researchers for various applications, including whole bacterial genome sequencing, complete plasmid sequencing, prediction of antimicrobial resistance, and metagenomic and microbiome analysis, among others (7–10). Globally, R9 ONT technology played a pivotal role in responding to the COVID-19 pandemic, providing crucial support to public health responses and authorities (11). The first ONT diagnostic assay, the LamPORE COVID-19, was developed and received CE marking in October 2020, with sensitivity and specificity exceeding 99.5% (12). With continuous ONT development and enhancement efforts, the R10.4.1 flow cells and associated kit were announced in late 2022, promising even greater sequencing accuracy.

The R10 technology introduces a new pore design featuring a longer barrel and dual reader head, which enhances consensus accuracy, particularly in homopolymer genomic regions. Additionally, duplex basecalling enables the immediate reading of the complement strand following the template strand, further enhancing sequencing accuracy. Bogaerts et al. (13) compared the R10.4.1 technology to R9 and Illumina whole-genome sequencing (WGS) for characterizing single-nucleotide polymorphisms (SNPs) in outbreaks caused by bacterial pathogens. The authors utilized data sets from Shiga toxin-producing *Escherichia coli* and *Listeria monocytogenes* outbreaks and employed an open-source workflow (prokaryotic awesome variant calling utility; PACU) for SNP phylogeny analysis. Their findings demonstrated that the results obtained with R10 were comparable to Illumina sequencing and exhibited greater accuracy than those obtained with R9. Consistent with these observations, Gordon et al. demonstrated the

high accuracy of ONT R10.4.1 in detecting quinolone resistance and its concordance with phenotypic antimicrobial susceptibility in a community cluster of multidrug-resistant (MDR) *Shigella sonnei* (14). Furthermore, another study recently revealed that assembling genomes and plasmids of eight MDR bacterial strains using solely ONT R10.4.1 closely matched the data generated with DNBseq-T7 (MGI) NGS, achieving a 99.99% identity (15). The authors also emphasized the instrumental role of ONT long reads in recovering complete plasmids and elucidating their structural complexity. The need for short reads to correct the ONT long reads' introduced insertions, deletions, and errors due to homopolymer regions were rendered unnecessary with the R10.4.1 technology, even when recovering complete bacterial genomes from activated sludge, rather than pure bacterial isolates (16).

Besides WGS applications, full-length 16S rRNA was also successfully recovered with high accuracy using ONT R10.4.1, exhibiting a significantly reduced error rate, particularly for deletions. This improvement enabled species-level identification of microbiota (17). Furthermore, viral sequencing with ONT R10 demonstrated high accuracy. Consistent with our unpublished data, Ratcliff et al. showed that the R10 chemistry enhanced sequencing accuracy for influenza homopolymer regions, including the cleavage site of the hemagglutinin gene (18). SARS-CoV-2 whole-genome sequencing accuracy was also enhanced with R10.4.1, with mutation analysis yielding results completely concordant with Illumina (19).

Bogaerts et al. (13) present a WGS SNP-based phylogeny for both Illumina and ONT R9 and R10 data, which they designated PACU. PACU was evaluated using the Shiga toxin-producing *Escherichia coli* and *Listeria monocytogenes* outbreak data. As the authors highlight, SNPs and variant callers have been developed, but predominantly for Illumina data set analyses. However, the release of ONT R10.4.1 was associated with the introduction of more accurate basecalling models and coupled with the duplex reads, recovering highly accurate raw reads was an outstanding improvement (20). With equivalent accuracy observed between Illumina and ONT R10.4.1 raw reads, both technologies are likely to yield similar SNPs when analyzed with a shared pipeline. The authors compared PACU for Illumina and ONT R9 and R10 and identified a large number of SNPs with the ONT R9 than those called with Illumina or R10. The R10 phylogeny was closer to Illumina than the R9 phylogeny. The authors emphasize that data generated by different chemistries can be analyzed with the same pipeline, facilitating a reproducible approach for calling variants. This unified approach is particularly attractive given the high accuracy of ONT R10, enabling laboratories collaborating on outbreak investigations using both Illumina and ONT R10 to share the same analysis pipeline. Overall, when genomic data are analyzed using different pipelines, it is crucial to evaluate the reproducibility of SNP calling, especially in outbreak investigations where SNP accuracy is paramount.

In conclusion, the ONT R10 represents a new era in ONT sequencing, combining long reads, sequencing flexibility, rapid solutions, comparative lower costs, and notably, a high level of accuracy. Its comparable sequencing accuracy to Illumina demonstrates the feasibility of using ONT R10 for applications that previously relied on Illumina-generated reads, such as filling gaps in genomes with homopolymer regions, insertions, or deletions. ONT R10 shows promise as a standalone approach for recovering complete genomes, metagenomes, and high-accuracy SNP identification.

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