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MINIREVIEW

Third-Generation Sequencing in the Clinical Laboratory: Exploring the Advantages and Challenges of Nanopore Sequencing

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ABSTRACT Metagenomic sequencing for infectious disease diagnostics is an important tool that holds promise for use in the clinical laboratory. Challenges for implementation so far include high cost, the length of time to results, and the need for technical and bioinformatics expertise. However, the recent technological innovation of nanopore sequencing from Oxford Nanopore Technologies (ONT) has the potential to address these challenges. ONT sequencing is an attractive platform for clinical laboratories to adopt due to its low cost, rapid turnaround time, and user-friendly bioinformatics pipelines. However, this method still faces the problem of base-calling accuracy compared to other platforms. This review highlights the general challenges of pathogen detection in clinical specimens by metagenomic sequencing, the advantages and disadvantages of the ONT platform, and how research to date supports the potential future use of nanopore sequencing in infectious disease diagnostics.

KEYWORDS nanopore sequencing, third-generation sequencing, infectious disease diagnostics

Infectious disease diagnostics currently involve an array of laboratory methods, including culture, serologic assays, nucleic acid amplification tests, antigen detection, and direct visualization. The complexity of diagnostic options requires nuanced understanding on the part of the clinician and careful assessment of a patient's clinical presentation and history to ensure appropriate test ordering. For culture-based methods, a growth amplification step is necessary that can take anywhere from a day to several weeks, depending on the type of pathogen causing infection. The likelihood of isolating a pathogen is compromised when fastidious organisms are present or when a patient is receiving antimicrobial therapy. Developing increasingly rapid, accurate methods to identify pathogens and characterize antimicrobial resistance (AMR) is a constant quest in the field of infectious disease diagnostics in order to improve patient outcomes. In the face of the complexity of current methods, metagenomic next-generation sequencing (NGS) offers the possibility of universal pathogen detection, enabling the identification of bacteria, fungi, viruses, and parasites with a single method directly from patient specimens (1, 2).

The advent of NGS in the early 2000s revolutionized the field of genomic research. NGS encompasses several different approaches to nucleic acid sequencing; however, they all utilize the same basic approach in which either DNA or RNA molecules are sequenced in a massively parallel manner (3). The initial preparation of samples for sequencing is often a technically laborious process wherein DNA or RNA has to be isolated, checked for quality metrics, and then put through a library preparation

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TABLE 1 Advantages and disadvantages of the 4 major sequencing platforms

Sequencing platform	Chemistry	Avg read length (bp)	Advantage(s)	Disadvantage(s)[a]
Illumina	Sequencing by synthesis; fluorescently labeled deoxynucleoside triphosphates	≤300	High accuracy	Short reads, high capital cost, long TAT
Thermo Fisher Ion Torrent	Sequencing by synthesis; detection of hydrogen ions	≤400	High accuracy	Short reads, high capital cost, long TAT
Pacific Biosciences	Sequencing by synthesis: SMRTbell replication	≥500	Long reads	High capital cost, variable accuracy, long TAT
Oxford Nanopore	Measures the changes in current as biological molecules pass through the nanopore	≥500	Long reads, low capital cost, short TAT	Low accuracy

[a]TAT, turnaround time.

protocol that can take hours to days to complete. Sequencing itself can then take anywhere from 1 to 6 days, depending on the platform used, the length of the reads, and the amount of data generated (Table 1). Illumina and Thermo Fisher offer short-read (100 to 400 bp) sequencing platforms, whereas Pacific Biosciences and Oxford Nanopore Technologies (ONT) offer long-read (>500 bp) sequencing (3, 4). Each platform offers its own advantages and disadvantages in terms of accuracy, efficiency, and cost. Regardless of the technology used, bioinformatics knowledge is required for data processing and analysis. Furthermore, turnaround time from nucleic acid extraction through final result reporting typically takes a minimum of 5 to 10 days.

NGS in the clinical setting has become widespread in personalized medicine due to its ability to characterize variants throughout the human genome. The Illumina and Thermo Fisher platforms are in widespread use to identify somatic mutations in cancer and to uncover the genetic basis of various inherited diseases (5, 6). Application of NGS to infectious disease diagnostics has been slower to evolve for a number of reasons. In this review, we focus on challenges for implementation of metagenomic sequencing for pathogen detection, why nanopore sequencing could overcome many of those hurdles, and how research performed to date supports the future use of ONT sequencing in the clinical laboratory.

NGS FOR INFECTIOUS DISEASE DIAGNOSTICS

The use of NGS for infectious disease diagnostics is being increasingly explored and adopted for select applications in the clinical laboratory (4, 7–12). Several approaches to pathogen detection with NGS can be undertaken (Table 2). Metagenomic whole-genome sequencing (mWGS), which analyzes all of the DNA or RNA in a given sample, offers an unbiased, hypothesis-free approach to detecting bacteria, viruses, fungi, and parasites. Aside from high cost, an additional consideration for performing mWGS is the abundance of human DNA in many clinical sample types such as blood and respiratory secretions (13, 14). Human contamination results in most sequence data belonging to the host instead of pathogen, making it more difficult to detect and characterize

TABLE 2 Current NGS approaches for infectious disease diagnostics[a]

Sequencing approach	Target	Advantage(s)	Disadvantage(s)
16S rRNA gene	Bacteria	Lower cost, targets and amplifies most bacteria present, low-complexity bioinformatics	Specific to bacteria, can still miss some species due to primer mismatches, bacterial ID only
18S rRNA gene/ITS gene	Fungi and some parasites	Lower cost, targets and amplifies most fungi present and some parasites, low-complexity bioinformatics	Specific to fungi and a subset of parasites, can still miss some species due to primer mismatches
Whole genome	Cultured organism	Provides in-depth coverage of a single organism with associated virulence and antimicrobial genes	Requires a cultured organism, high-complexity bioinformatics
Metagenomic whole genome	Unbiased	Sequence any organism that is present, can analyze entire genomes, ID of virulence and AMR genes	High cost, high-complexity bioinformatics, host contamination

[a]ID, identification.

infectious agents. Furthermore, analysis of any samples containing human reads should be done on a secure, HIPAA (Health Insurance Portability and Accountability Act of 1996) compliant platform to ensure protection of genomic information. An alternative approach for bacterial pathogens is targeted, 16S rRNA gene sequencing (15, 16). The 16S rRNA gene has been the most commonly used region for bacterial detection and identification because it exists in almost all bacteria, because it has conserved regions that enable universal primer design, and because smaller portions of the gene, termed variable regions, can be sequenced, as opposed to the entire gene (1,500 bp) (17). Polymorphisms within the variable regions are what distinguish different species of bacteria with $<$2% variability differentiating closely related species (18). Similarly, internal transcribed spacer (ITS) sequencing can be utilized to target fungi for sequencing (19). Advantages of targeted methods over mWGS include increased sensitivity, decreased cost, and decreased complexity of computationally intensive bioinformatics. However, 16S rRNA and ITS sequencing primers will not pick up every bacterial or fungal species due to variation in nucleotide composition at primer binding sites (19, 20). In addition, 16S rRNA gene and ITS sequencing can provide only taxonomic identification while mWGS can also provide genomic data on virulence, AMR, metabolism, and strain typing that may be of clinical or epidemiologic utility.

To date, regardless of the sequencing approach, logistical hurdles for implementation include the high cost for capital investment and consumables, availability of trained/experienced laboratory personnel and lack of prospective clinical outcomes data to justify increased laboratory costs. In addition, there are numerous possible approaches to wet lab work flow all of which must be optimized, including storage and processing, nucleic acid isolation protocols, controlling for environmental contamination, sequencing library preparation methods, and choice of sequencing platform. The wet lab protocols to be used are highly dependent on the sample type, the type of organism(s) targeted, the amount of nucleic acid isolated, and the sequencing approach. The overall design of each step in the process can influence final results and even the ability to detect certain pathogens (21).

In addition to the variability of wet lab protocols, the bioinformatics for data handling and interpretation can be resource intense. Bioinformatic analysis requires highly trained staff, valid analysis tools, including the reference database, the computational infrastructure, and the creation of standardized procedures. Each of the four sequencing platforms (Illumina, Thermo Fisher, Pacific Biosciences, and ONT) require their own initial data processing steps and quality control metrics. For bioinformatics, the tools that have been developed in the research community for short-read data are not feasible for long-read data. Data interpretation adds an additional level of complexity. In clinical specimens it can at times be difficult to distinguish normal microbiota or colonizers from pathogens since certain organisms can be either (1). Thresholds of detection for clinical relevance for certain organisms in certain specimen types need to be established before routine use of NGS for common specimen types is possible. The true complexities of how different bioinformatics pipelines influence data analysis is beyond the scope of this review; however, there is no one "correct" way to handle sequence data.

Due to the multitude of options available for everything from sample collection through final interpretation of results, efforts are under way to standardize key parts of the process for NGS in infectious disease diagnostics. The recent release of the FDA-ARGOS reference database signified major headway in the direction of standardization (22). However, a plethora of challenges remain for how to establish protocols from sample collection all the way through final data analysis (7). It is likely that standardized protocols will need to be developed for each sample type, sequencing approach, and sequencing platform. The inclusion of negative and/or no template controls should be mandatory to aid in identifying any possible false-positive results. One of the few commonalities across platforms and sample types will be the database used and specific thresholds (such as read depth and genome coverage) used during data analysis, parameters which are already being explored (10, 11). Also important for

validation across different laboratories will be proficiency material, such as the use of commercial standards from companies such as the American Type Culture Collection that contain mixed microbial communities.

Another barrier to implementation of NGS in infectious disease diagnostics has been the time it takes to complete all the steps for NGS. In general, it can take a minimum of 1 week to prepare samples, sequence libraries, and analyze data once standardized protocols are put into place. Altogether, this turnaround time relative to conventional methods has limited the clinical relevance of NGS results for patient care decision-making. Because of this, many NGS applications in the clinical microbiology laboratory target situations where a rapid answer is not necessary. This includes outbreak surveillance, infection prevention, and WGS of antibiotic-resistant isolates (23–27). Rapidly evolving NGS technology, however, may soon address many of the challenges discussed thus far.

NANOPORE SEQUENCING

While NGS platforms from Illumina, Thermo Fisher, and Pacific Biosciences have revolutionized biomedical research, a novel approach to NGS using nanopore technology has the potential for use in the clinical lab in the near future. Oxford Nanopore Technologies (ONT) first introduced the MinION platform to the research market in 2014. MinION is different from other platforms because it utilizes nanopores for sequencing (28). It does not take a sequencing-by-synthesis approach; instead, an ionic current is passed across the flow cell during sequencing, and the different nucleotide bases are distinguished by the changes in current as they pass through the nanopores (28). Sequencing with the MinION platform requires minimal capital cost compared to the Illumina, Thermo Fisher, and Pacific Biosciences platforms and can be utilized both in the laboratory and out in the field. Able to fit in the palm of your hand and connected to either ONT's "MinIT" computer module or to any computer with a USB connection, MinION permits direct, electronic analysis of single molecules in real time. It can be used to analyze DNA and RNA for a range of applications, including personalized medicine, agriculture, and scientific research.

Although nanopore sequencing is able to produce reads of up to 2 Mb in length (29), the biggest drawbacks to date have been a lower throughput of sequence data and a high error rate (approximately 10%) with their 9.4 and earlier-version flow cells that use 1D chemistry (30). The 9.5 flow cells utilize $1D^2$ chemistry and have been reported to achieve 99% accuracy in base calling of the 16S rRNA gene of a group of commonly identified sepsis-causing organisms (31). Furthermore, the new version 10 flow cells utilize a new type of nanopore, and ONT claims this flow cell can deliver up to 99.99% base-calling accuracy. Regardless of these recent improvements, the lower accuracy in base calling with earlier versions of the MinION flow cells has not limited the use of nanopore sequencing in the infectious disease research setting. Validation of the accuracy of nanopore sequencing long reads has been achieved by the inclusion of parallel sequencing with Illumina. In addition, long reads generated by ONT have been used to fill in the gaps in unfinished genomes sequenced on short-read platforms (32–37).

The advantages of nanopore long-read sequencing are numerous. No other platform allows for real-time analysis while sequencing is ongoing, which has been one of the major drawbacks for infectious disease diagnostics with other types of NGS technology. A MinION flow cell can accommodate numerous concurrent patient samples and generates between 4 and 8 GB of data. It can then be reused up to ten times to maximize use of a single flow cell, which in turn can lower costs. This is advantageous for instances when only a few samples are available for sequencing and/or low read depth is required. Another advantage of nanopore sequencing is the time required for sequencing library preparation. If enough DNA is available (400 ng in 7.5 μl) the Oxford Nanopore rapid barcoding kit allows for a 10-min library preparation before loading the flow cell. Performing a sequencing run and obtaining primary acquisition of data are done with the graphical user interface program MinKNOW.

MinKNOW is used for selecting run parameters, tracking platform chemistry, and producing FAST5 files (raw signal data files). Users have the option of using MinKNOW for also producing FASTQ files, a method which then uses a data processing toolkit called Guppy to convert FAST5 files to FASTQ.

Alternatively, if users have bioinformatics expertise, there are a number of tools available for data analysis that aid in improving base-calling accuracy (38). The Guppy toolkit can be downloaded separately and used to process FAST5 files through command line interface on their computer. Utilizing Guppy in this manner offers the user several algorithms for base calling, which can improve raw read accuracy by upwards of 3%. The Guppy toolkit also includes downstream analysis components to allow for demultiplexing, adapter trimming, and alignment. There are also a number of higher complexity tools available for users looking to do more than simple taxonomic classification. Those developed by ONT can be found on their github page (https://github.com/nanoporetech). Available tools, among others, include medaka, tombo, pomoxis, and nanopolish, which are used for sequence correction, identifying modified nucleotides from raw sequencing data, genome assembly, and error correction in genomic assemblies, respectively (39–42).

The stated goal of ONT has been to enable the analysis of any living thing, by any person, in any environment. To support that goal, ONT created Metrichor, a branch of the company which has developed graphical user interface tools as part of their EPI2ME platform for users with limited bioinformatics knowledge (https://epi2me.nanoporetech.com). The apps under EPI2ME allow users to perform a number of different analyses, including demultiplexing, adapter trimming, and alignment to reference genomes. Additional options for microbiological specimens include taxonomic classification (using the "What's in my pot?" app), AMR gene ID, and direct alignment to a reference genome. Overall, these apps allow a user of ONT sequencing to process their data without needing to run software through the command line interface on their computer, an option not readily available from other platforms.

In the clinical laboratory, the most advantageous and readily applied use of nanopore sequencing will likely be in infectious disease diagnostics. As already discussed, it is an appealing option due to rapid library preparation and sequencing and user-friendly bioinformatics. Further addressing concerns over the cost for NGS, ONT is working to reduce the cost of a sequencing run with the introduction of the Flongle in March of 2019. The Flongle is an adapter (flow cell dongle) for MinION that performs sequencing on smaller, single-use flow cells. At a cost of approximately $100, it is significantly less expensive than the MinION flow cells that currently cost $900. Sequencing with the Flongle allows the user to generate approximately 1 GB of data in approximately 24 h and is therefore a cost-efficient and rapid option for smaller sequencing experiments. This is ideal for infectious disease diagnostics where optimizing antimicrobial therapy depends on rapid turnaround time for pathogen detection, identification and AMR characterization.

If a user in a clinical laboratory wants to perform taxonomic classification to a more expanded and secure database than the "What's in my pot?" app in EPI2ME, an alternative fast and easy option to explore is the One Codex platform (One Codex, San Francisco, CA). One Codex is an online platform that offers taxonomic analysis, AMR prediction, and direct alignments for any microbiology-based samples (43). The One Codex general database includes >61,000 bacterial, >48,000 viral, >1,800 fungal, >1,900 archaeal, and >200 protozoan genomes. It offers a targeted locus database for 16S, 5S, 23S, *gyrB*, *rpoB*, 18S, 28S, and ITS gene analysis. The AMR panel includes >200 genes, and the analysis includes percent identity, percent coverage, and read depth to each gene. One Codex also offers a HIPAA-compliant account, making it a better option than analysis with EPI2ME apps, which do not offer the level of security necessary for uploading patient samples. One Codex is one possible option for fast, secure analysis for laboratories who do not want to invest the time or resources for in-house microbial classification pipelines. Other commercially available platforms for bioinformatic analysis, such as Diversigen (44), CosmosID (45), and Real Time Genomics (46), are addi-

tional options for clinical laboratories unable to build and maintain taxonomic databases.

NANOPORE SEQUENCING IN INFECTIOUS DISEASE RESEARCH

Despite several hurdles that ONT still faces, a number of research publications have illustrated just how versatile and applicable nanopore sequencing is. Not only could the MinION be taken directly to the bedside of a patient, nanopore sequencing has been used extensively outside the laboratory in many different environments. Researchers have taken the MinION to the Arctic for characterizing permafrost ice wedge microbial communities (47), to the military training grounds at NATO's Counter Terrorism and Technology Centre in Alberta, Canada, for detection of biological agents (48), and even on the International Space Station to determine how the sequencer performs in space using viral, bacterial, and mouse DNA (49). The MinION has also been used in West Africa during an ongoing Ebola epidemic, where having genome sequencing of the virus on-site allowed for real-time surveillance of the outbreak (50). In these types of situations where resources are scarce and a virus such as Ebola virus can rapidly evolve, having nanopore sequencing available for genomic surveillance can greatly aid in pathogen identification and in monitoring patient responses to vaccines and treatments.

The MinION platform has also been used for research in a number of clinically relevant infectious disease applications. Bacterial and fungal identification of clinical isolates using 16S rRNA and ITS gene sequencing has been successful (31). Researchers were able to obtain 99% accuracy of the 16S rRNA and ITS genes using the 9.5 version flow cells, making the option of targeted sequencing feasible for clinical laboratories on the MinION. Nanopore sequencing has also been used to successfully identify pathogens in clinical cases where identification of the infectious agent can be difficult (51, 52). mWGS with nanopore sequencing was carried out on seven DNA isolates from resected heart valve tissue obtained from patients diagnosed with infective endocarditis (51). Although all seven samples were determined to be culture negative by traditional microbiology testing, species identification of pathogens, which included *Streptococcus*, *Coxiella*, and *Bartonella* spp., was attained in all cases. Prosthetic joint infections, similar to endocarditis, can also be difficult to properly diagnose and treat (53). In a recent study, nine samples, seven culture positive and two culture negative, obtained from sonication fluid were sequenced by mWGS on both the Illumina and ONT platforms (52). Results from the ONT platform corresponded with metagenomics Illumina MiSeq sequencing and culture-based methodologies with the exception that one sample had better species resolution with ONT compared to Illumina sequencing. Furthermore, two samples were found to have an additional species called with the nanopore sequencing pipeline compared to culture-based methods, indicating that ONT sequencing could provide further sensitivity for microorganism detection. Nanopore sequencing can also be applied in cases where a pathogen identified by normal methodologies requires rapid confirmatory testing. Targeted 16S rRNA gene sequencing with a MinION flow cell was performed on a blood-culture isolate obtained from a patient with meningitis (54). Taxonomic analysis confirmed the infectious agent was *Campylobacter fetus*, a zoonotic pathogen that rarely causes meningitis in humans.

Not only is pathogen identification important in clinical laboratories, but rapid and accurate AST results can significantly impact time to effective antimicrobial therapy. Long-read nanopore sequencing can identify the presence or absence of resistance genes, from which the phenotype of resistance can be inferred. A proof-of-principle study of 40 *Klebsiella pneumoniae* blood isolates evaluated different data analysis methods for AST prediction, including a real-time analysis approach to identify AMR genes and a nanopore assembly approach (55). That study found 77% agreement with phenotypic methods for a real-time nanopore analysis and 92% agreement with an assembly nanopore approach. Importantly, if employed in real time on a patient isolate, these methods would have hypothetically shortened median time to effective antimicrobial therapy by 20 and 26 h, respectively. In other less clinically oriented studies,

extensive characterization of the genomic context of AMR determinants has been elucidated in *Escherichia*, *Salmonella*, *Klebsiella*, and *Enterococcus* (56–60). Complete plasmid sequences, which are difficult to assemble with short-read platforms, have been resolved using ONT on a host of *Enterobacteriaceae* species (33, 59). The long reads generated by nanopore sequencing allowed for detection and mapping of mobile AMR elements in a multidrug-resistant strain of enteroaggregative *Escherichia coli* (57). Despite high error rates, a 2015 study using the earlier iteration of the MinION flow cell looked at AMR in four *Enterobacteriaceae* species, an *Acinetobacter baumannii* isolate, and a methicillin-resistant *Staphylococcus aureus* and found that with even coverage across the genome a specific subset of AMR genes can be accurately called (61). The number of reads necessary to confidently call AMR is not yet well defined. One recent study argues that only one relevant read is necessary to call AMR for a particular drug due to a lower threshold being allowed for long-read sequencing (51), while others have used a threshold of 10 reads (55). In addition to AMR, nanopore sequencing can also provide other genomic data of clinical use. For example, when performing WGS on clinical isolates with both ONT and Illumina sequencing, identification of mutations that may lead to escape of vaccine-induced immunity have been elucidated for *Bordetella pertussis* (36, 62).

Nanopore sequencing has also been used to identify clinically relevant viruses, often with extensive coverage of entire viral genomes. A 2015 study using an earlier version of the MinION correctly characterized three distinct strains of poxviruses despite an error rate of approximately 30% (63). Another study reported on the detection of chikungunya virus, Ebola virus, and hepatitis C virus from blood samples, with a total turnaround time from sample to report as $<$6 h (64). Whole genomes of influenza viruses have been sequenced using ONT, demonstrating the use of this technology to track emerging strains of influenza that could potentially be utilized for better vaccine preparation (65).

NEXT STEPS FOR NANOPORE SEQUENCING IN THE CLINICAL LABORATORY

Although application of nanopore technology in the clinical laboratory is still in the research phase, there is significant potential for its use in personalized medicine. The size of the sequencer and cost for utilization makes it ideal for any size clinical laboratory, with the main caveat that appropriately trained personnel are available to carry out method validation, perform nucleic acid extraction, carry out library preparation and sequencing, and analyze the data. Not only would nanopore sequencing be useful for characterizing microorganisms for infectious disease diagnostics, it could be a useful tool for monitoring the human microbiome. The field of microbiome research has exploded over the past 10 years and may become important in clinical medicine in the future (66, 67). For example, the gut microbiome could be monitored over time in individuals undergoing repeated or long-term antibiotic therapy, which can lead to dysbiosis in the gut and other gastrointestinal disorders (68, 69). Nanopore sequencing may also be helpful in characterizing the lung microbiome of individual patients with cystic fibrosis and then diagnosing infection during a pulmonary exacerbation. These are just a few examples of how nanopore sequencing can be utilized in medicine, and the possibilities extend far further than what is mentioned here.

CONCLUSIONS

NGS proof-of-principle assays have been widely used in research to identify and characterize infectious disease agents. Numerous challenges and unknowns limit many clinical laboratories from implementing this technology in-house. More outcomes research, as well as the use of standardized databases, appropriate proficiency materials, and validated thresholds for reporting detected pathogens, will be of utmost importance. The correlations between AMR genotype and phenotype are still not completely understood, which can lead to difficulties in data interpretation. In addition, training and maintaining staff with the appropriate expertise may be a major hurdle for some diagnostic laboratories. It is unlikely to completely replace any current method of

conventional diagnostic testing in the near future. Despite these challenges for implementation, mWGS is a promising tool that could provide clinically relevant information such as universal pathogen detection and AMR prediction in a single assay.

Although many platforms for NGS exist, third-generation nanopore sequencing offers many solutions to the current problems of using mWGS for infectious disease diagnostics. It has been successfully utilized for pathogen detection, AMR prediction, and characterization of mixed microbial communities. As improvements continue to be made toward higher accuracy and robust performance, the clear advantages in cost, turnaround time, and user-friendly bioinformatics will likely make it a viable option in the near future for clinical laboratories wanting to implement NGS in-house for infectious disease detection and mWGS.

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