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Nanopore Sequencing for Diagnosis and Resistance Profiling of Pathogens

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Abstract

Effective treatment and preventive measures of infectious diseases demand rapid and accurate identification of causative agents. Oxford nanopore MinION™ is a commercially available portable, convenient, relatively fast, and cost-effective DNA sequencer providing sequencing data in real-time. MinION™ utilizes the base-specific fluctuations due to blockage of a nanopore and ultimately transforming it into DNA sequence information. Nanopore MinION™ has been used worldwide to deliver new insights and real-time results for a broad range of applications, which include epidemiological surveillance programs, field diagnostics, microbiome identification. Long read Oxford Nanopore MinION™ whole-genome sequencing has been accurately used for sequence typing and determining antibiotic resistance profiles of various pathogens. Oxford nanopore MinION™ sequencing offers a vast potential for cost-effective, rapid whole-genome sequencing for pathogen diagnosis and resistance identification in real-time.

Introduction

The technique of determining the order of the nucleotide bases in the nucleic acid molecule DNA is called DNA sequencing. First-generation DNA sequencing technologies, i.e., the Maxam-Gilbert method and Sanger sequencing method, were invented in the 1970's. Sanger sequencing method using fluorescent tags to label the DNA molecules was most commonly used with the help of automated sequencing instruments. Later on, with the emergence of second-generation techniques, sequencing became much faster, with high throughput capacity and more cost-effective as compared to first-generation technologies. Nowadays, the recent development of single-molecule sequencing technologies (third-generation sequencing) offers many advantages over the second-generation sequencing by- (i) generating ultra-long label-less reads, (ii) rapid library preparation, (iii) requiring only a small amount of DNA with low cost for a single run and (iv) real-time data acquisition (v) portability of the sequencer. During the last decade, whole-genome sequencing (WGS) got much more attention to be utilized in public health practice, such as in the cholera epidemic in 2010 and the international outbreak of *E. coli* O104:H4 and for testing of epidemiological associations in hospital-acquired infections. Nowadays, nanopore sequencing, a third-generation sequencing technique, is generating a lot of interest in the scientific community for identification and resistance profiling of microorganisms. Of late, the release of the MinION (Oxford Nanopore Technologies, Oxford, UK), a novel portable real-time NGS sequencer, allows rapid diagnosis with inexpensive sample preparation even in low-throughput laboratories. The MinION sequencing device generating long reads is critical for the discovery of co-localized antimicrobial resistance genes and their flanking nucleotide sequence in the

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host bacteria and infectious disease diagnosis. This portable nanopore sequencing device MinION is commercially available in the market for sequencing DNA at a scale of single-molecule (Mikheyev and Tin, 2014).

Principle of Nanopore Sequencing Technology

The idea of using nanopore sensors for DNA sequencing was independently proposed by Church *et al.* and Deamer and Akeson in the 1990s. After some years, in 1993, Deamer, Branton, and Kasiannowicz demonstrated the translocation and detection of DNA through α-hemolysin (α- HL) nanopore, a toxin produced by *Staphylococcus aureus.* Nanopores, in general, have many potential applications such as the analysis of ions, DNA, RNA, peptides, proteins, drugs, polymers, and macromolecules. The technology utilizes nanopores roughly 1 nm in diameter embedded in a biological membrane or solid film integrated with a semiconductor-based electronic system for sequencing of nucleotides. DNA to be sequenced is prepared according to a protocol and includes attaching a leader adapter and motor proteins to one strand of DNA (Figure 1). Motor protein unwinds dsDNA to ssDNA to enable it to pass through the nanopore. More specifically, each nanopore has a DNA polymerase enzyme as motor and modified tagged nucleotides corresponding to each base. The conduction of ionic current through the electrolytic medium occurs when DNA passes through the nanopore in the presence of an electric field. The pore of the nanochannel, when blocked by a biomolecule (e.g., a negatively charged DNA molecule), it stops the flow of the current, with each base producing specific amplitude and duration of blockades. These base specific fluctuations or electric signals are used to determine the physical and chemical properties of the target bases and ultimately getting transformed to DNA sequence information (Bayley, 2006).

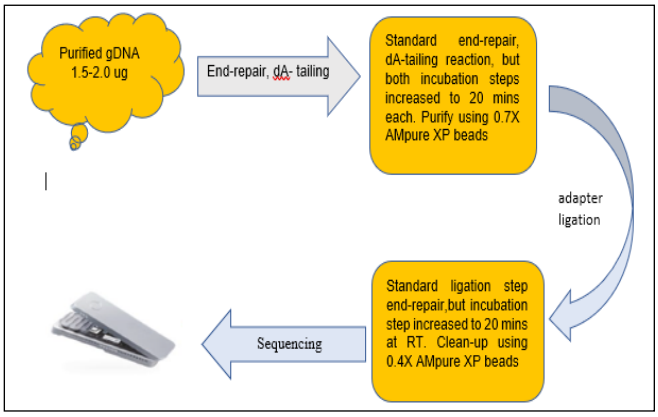

Figure 1: Library preparation and WGS workflow for sequencing by Nanopore MinION technology. (Source: WHO/CDS/TB/2018.19)

Diagnosis and Resistance Profiling of Pathogens

Nanopore sequencing technology is advancing rapidly, promising to be a potential portable sequencing technique for routine surveillance and in many other fields in the future. Oxford Nanopore MinION has been utilized worldwide to deliver new insights and real-time results for a broad range of applications (Figure 2). The comparison of this device with other commercially available sequencing instruments is given in Table 1.

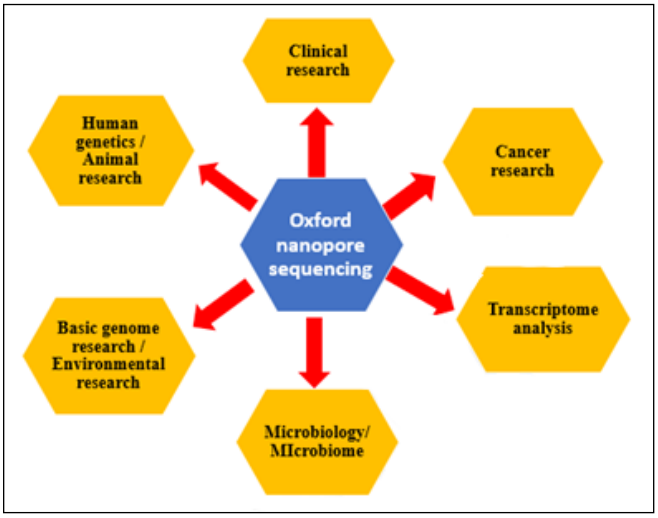

Figure 2: Various applications of Oxford MinION Nanopore Technology

Table 1: Comparison of commercially available NGS instruments

Sequencing platform	Sequencing kit	Number of samplesper run	Typical run output	Typical run output	Sequencing run time
Illumina MiSeq	MiSeq v2 (2x250bp)	54	$22.41	7.5-8.5GB	39 hr
Ion Torrent	Ion 318 Chip v2 (400 base)	13	$48.75	1.2-2GB	7.3 hr
Oxford Nanopore	Ligation Sequencing kit 1D	22 54	$42.86 $16.67	2-3 GB 4-8 GB	6 hr 48 hr

(Source: WHO/CDS/TB/2018.19)

Effective treatment and preventive measures of infectious diseases demand rapid and accurate identification of causative agents. Time-consuming and less sensitive techniques for diagnosis impede various control-based efforts for infectious diseases. Compared to PCR and ELISA based assays, nanopore sequencing offers excellent potential to diagnose rare, novel etiological agents, drug resistance profiles of pathogens in real-time. Nanopore metagenomics has been found promising to replace the less-sensitive and time-consuming culture techniques to diagnose bacterial lower respiratory infections and the identification of viruses like Ebola, Chikungunya, Hepatitis C, and *Haemophilus influenzae* in humans (Figure 3).

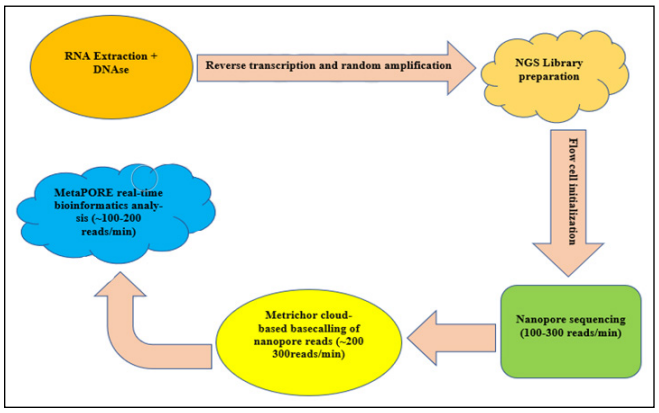

Figure 3: Metagenomic sequencing workflow for MinION nanopore sequencing. Greninger *et al*. (2015)

The identification of pathogens directly with no culture involved from urine samples in a period of 4 hours was demonstrated using MinION. Generating data for the identification of pathogens in real-time, along with the antibiotic resistance profiles, may potentially help to improve health strategies in clinical settings and the better use of specific antibiotics. MinION sequencing platform was demonstrated to rapidly identify particular pathogens from the mixed gut microbiome in real-time along with their corresponding AMR profiles in preterm infants. Interestingly, the great adaptability of the nanopore sequencing approach allows its usage with various other techniques. LAMP assay for amplification along with MinION sequencing was used for the rapid detection of malarial parasites in blood and Cutaneous leishmaniasis. Nanopore sequencing is emerging as a promising approach to diagnose pathogens and identify resistance patterns in human clinical settings by genomic analyses, particularly in the developing world, with limited resources for testing and surveillance of infectious diseases. Besides this, several reports in the same line exist in veterinary medicine. A study aimed at validating nanopore sequencing for routine diagnosis of canine distemper virus (CDV) revealed MinION a rapid, inexpensive, accurate enough for diagnosis as well as surveillance of CDV in field conditions. A quick and reliable protocol for Serotyping using a MinION device to allow point-of-need Foot and mouth disease (FMDV) serotyping within five hours, which can be quickly followed in endemic countries, has also been developed. A metagenomics study using MinION was reported to identify enteric pathogens in swine. In fisheries and aquaculture, rapid genome-wide sequencing of fish viral pathogens using nanopore sequencing on the MinION platform has been demonstrated for two diseases causing agent; salmonid alphavirus (SAV) and infectious salmon anemia virus (ISAV) (Greninger *et al.*, 2015).

Furthermore, real-time whole-genome sequencing of emerging viruses can greatly benefit field diagnostics and epidemiological surveillance programs. Long reads obtained with MinION direct sequencing of avipox viruses from lesions without enrichment or isolation steps were found easy to assemble, requiring less bioinformatics capacity and processing time compared to earlier sequencing methods. These advantages accord nanopore sequencing enough scope for the assembly of genomes, which was challenging previously with the traditional short-read sequence by synthesis methods. The role of nanopore sequencing has also been extended for microbiome characterization and pathogen identification in plants. The portable nanopore sequencer has been reported to rapidly characterize a broad range of pathogenic fungi with the associated microbiota and accurate detection of viral diseases of wheat crops. This has enormous potential to enhance agricultural biosecurity and reduce crop losses due to emerging diseases.

MinION sequencing offers potential advantages for the detection of resistance genes on plasmids. The reporting of individual genes can replace traditional testing for AMR detection and help in guiding clinical management in many cases. A workflow for resistance gene detection on the plasmid was found significantly quickly as compared to the traditional susceptibility testing in various clinical isolates of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and *K. pneumoniae*. The current short read fragmented sequencers have severe limitations for assemblies and downstream analyses. Long read Oxford Nanopore MinION whole-genome sequencing was accurately used for sequence typing and determining antibiotic resistance profiles of bacterial model pathogen *Streptococcus suis* and the cause of anti-malarial drug resistance in parasite *Plasmodium falciparum.* Sea-lice *(Caligus rogercresseyi)* causes severe and welfare and economic concerns to the salmon fish sector in Chile. The role of microbiota associated with this ectoparasite life strategy was recently investigated by nanopore MinION sequencing by characterizing the associated microbiota. The study will serve a foundation to investigate the putative role of sea lice as vectors for fish pathogens and as reservoirs for antibiotic-resistant genes.

Conclusion

Traditional sequencing techniques have carried reliable identification of microbial species. However, the library preparation for such sequencing techniques is laborious and time-consuming, often takes days to weeks. Minion is a

small, handheld device (90 g) which can be connected to a computer system. Oxford Nanopore sequencing is a relatively fast and convenient technique. Depending on the kit and the protocol, the library preparation takes from 10 minutes and 3 hours.

Additionally, the rapid identification by nanopore sequencing allows the detection of pathogens in real-time to the species level at lower costs. AMR profiling with PCR and short-read sequencing has its disadvantages. Identification of *de novo* mutations and repetitive sequencing insertions with PCR and short-read sequencing respectively is not suitable. Nanopore sequencing offers a solution to this problem by generating ultra-long reads and resolving the arduous, repetitive regions. Rapid and cost-effective oxford nanopore real-time AMR identification will help to prevent further incidences of resistance spreading.

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