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IMPLICATION OF NANOPORE SEQUENCING TECHNOLOGY FOR PATHOGENIC DIAGNOSTICS: A MINI-REVIEW

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Abstract – *DNA sequencing is the most critical technique to explore and study genomic data of human beings and surrounding environments. The history of DNA sequencing has rapidly grown since the first-generation DNA sequencing in 1977. Until now, emerging high-throughput sequencing technology has recently revolutionized omics areas, including genomics, transcriptomics, metagenomics, and metabolomics for the development of modern molecular biology. Besides the short-read sequencing method (Illumina platforms), the long-read sequencing technologies (including Pacific Biosciences and Oxford Nanopore Technologies) have been developed with the advantages of larger read sizes, portable devices, and diverse preparation kits. Consequently, the number of biological databases and study-related nanopore sequencing technologies has grown fast. This review paper summarized the principle and fundament of nanopore sequencing regarding their advanced applications in pathogenic diagnostics of different hosts. The most recent application of nanopore sequencing in the diagnostics of infectious diseases is highlighted and discussed to provide insights into the novelty of nanopore sequencing technology and its future perspectives.*

Keywords: DNA sequencing, infectious diseases, Oxford Nanopore Technology, pathogens diagnostics.

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I. INTRODUCTION

In 1977, a new method for DNA sequencing, commonly called Sanger sequencing method, was reported and has been widely used until now [1]. Although the Sanger sequencing method exhibited a high accuracy (> 99.99%) and could produce long reads (> 600 bp), the demand for large amounts of data for genomic research triggered the emergence of new sequencing methods. Consequently, high-throughput sequencing methods were established, which could generate large volumes of genomic data with high accuracy, rapid processing, and cost-effective features [2]. The high-throughput sequencing platforms such as Illumina, Roche/454 pyrosequencing, Ion Torrent, Pacific Biosciences (PacBio), and Oxford Nanopore Technologies (ONT) revolutionized genomic studies of different organisms [3]. The short-read sequencing platform (i.e., Illumina) has been currently used in different research fields; however, genomes containing large duplication, deletion, and repeat elements cannot be fully assembled using short-read sequencing data and they require a complement of long-read sequencing data to be completed [4]. The PacBio and ONT platforms can perform the sequencing process without PCR-based library preparation and can produce long reads (longer than 10 kb with the PacBio system and up to 4000 kb with ONT devices) [5]. Although the PacBio platform exhibited higher accuracy (> 99%) than that of ONT (> 98%), the cost for the former is much more expensive than the latter. Both PacBio and ONT platforms have disadvantages and benefits but they are useful for the study of transcriptomics, DNA barcoding, long cell-free DNA, and genomics [6–10]. Due to the features of portable devices, affordable prices, diverse

kits for library preparation, and effective analysis workflows compared to other high-throughput sequencing technologies, the applications of ONT platforms have significantly increased [11]. The initial concept of nanopore sequencing was proposed through a series of studies and results of the nanopore and associated motor proteins in laboratories in the 1990s (Figure 1) [12, 13]. A notable study is the report of a pore-forming protein with a metal-actuated switch by Walker et al. [14]. The assembly ability of staphylococcal α-hemolysin to form hexameric pores in lipid bilayers leads to the idea that nanopore protein membranes can be used for biosensing cations and molecules [14]. Accordingly, several initial experiments on transmembrane were done by Deamer et al. [12] who filed patent applications for nanopore sequencing. The first DNA transport through α-hemolysin nanopore was proposed in 1996. The Mycobacterium smegmatic porin (MspA) was then explored and Jens Gundlach optimized MspA nanopore for better sensing of single molecules of DNA. In 2005, a nanopore achieved single-nucleobase discrimination among nucleobases in DNA strands, and ONT was released by Hagan Baley and colleagues. Then, the commercial MinION was successfully released in 2014. The invention of ONT provides a simple technique for genome sequencing of microbial, plant, animal, and human due to their long read with greater overlaps, leading to more advances in the de novo genome assembly implementation [15].

In the past decades, outbreaks of several diseases-based pathogens such as SARS, Ebola, and Zika virus required the development of innovative methodologies and techniques for efficient analysis in clinical practice and research. NGS, including nanopore sequencing, has rapidly developed and emerged as a powerful tool for identifying, characterizing, and monitoring pathogenic bacteria, fungi, and viruses. Initially, this review introduces the fundament and development of nanopore sequencing technology. Then, a summary of recent progress and implementation strategies for the application of nanopore sequencing in terms of pathogenic diagnostics in infectious diseases are described.

II. PRINCIPLE AND MECHANISMS OF OXFORD NANOPORE SEQUENCING

A. Nanopore-based biosensor system

The principle of a nanopore-based sensing system is based on the behavior of individual RNA/DNA molecules that can pass across the phospholipid bilayer membrane through protein ion channels by electrophoresis. In the biosensor system, the nanopores are typically located and impropriated in a phospholipid bilayer membrane. The membrane that contains nanopores separates two electrolyte-filled reservoirs. Ionic current can be generated when an electrical potential is introduced to two electrodes placed on opposite sides of a bilayer [16]. DNA owning a negative charge can be driven to translocate across the nanopore via the introduction of positive potential to the electrode on the opposite side of the bilayer membrane [17]. The internal diameters of nanopore-based α-hemolysin and nanopore-based MspA are 1.4 – 2.4 nm and 1.2 nm, respectively [18, 19]. MpsA is more narrow and stable than the channel of α-hemolysin, leading to its higher single-nucleotide resolution [13]. The changes in electrical current by translocation of nucleic acid through protein nanopores are monitored and encoded in the nucleic acid sequence. The translocation depends on several factors such as electrical potential, engineered nanopore, and the properties of nucleic acid fragments. The application of motor protein can help to improve the data quality and translocation kinetics [20]. Implication strategies of nanopore sequencing require a high throughput signal and accuracy. Bacteriophage phi29 DNA polymerase (phi29 DNAP) significantly improved DNA ratcheting through the nanopore [21]. The high affinity and processivity of phia29 DNAP allow its complex with DNA substrate across the α-hemolysin nanopore in an electric field.

ONT released the first nanopore sensing device, MinION, and commercialized it in

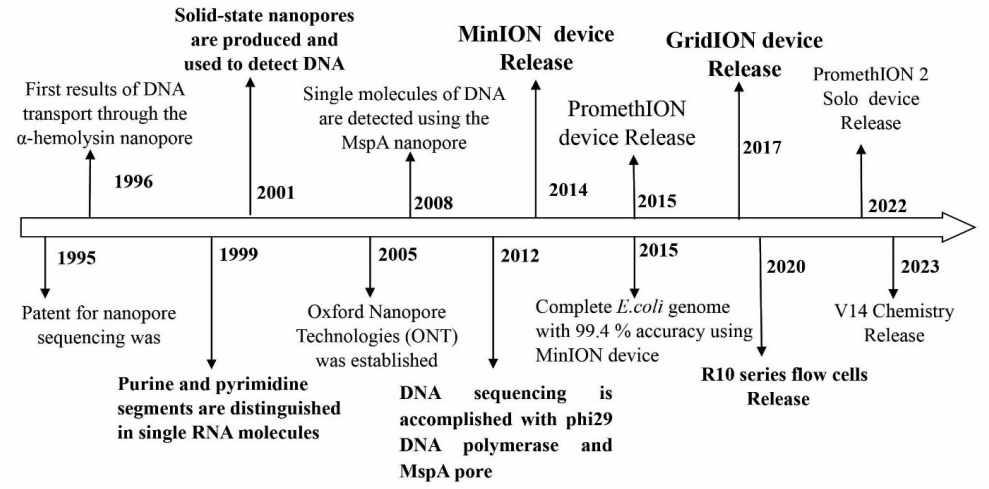

Fig. 1: The history of nanopore sequencing method modified from Ref. [12]

2015 [12]. ONT has successfully developed its nanopore sensing platforms GridION and MinION. The advancement of MinION devices is small, portable, and can plug directly into a USB3 port. MinKNOW, a specialist software, has been developed for MinION operating systems involving data acquisition, real-time analysis and feedback, data streaming, and ensuring the platform works correctly [22]. The ability of long-read ONT sequencing is developed cost-effectively and offers a wide range of applications in omics, especially for whole genome sequencing. In comparison, ONT and PacBio can perform long reads over 1000 bp, and Illumina can obtain a read length of up to 300 bp [23]. Long reads advance in the study of elucidation of genomic variations, repeat regions, phasing, and transcript isoform resolution. Practically, ONT has attempted to continuously improve their platforms (Flongle, MinION, GridION, PromethION), pore type (R9.4.1 and R10 series), kits (Q20+ and ultra-long kit), and algorithms and analysis (Bonito, MinKNOW, Guppy, and Dorado). MinION (Figure 2A) is a portable device with a weight of 87 g and small dimensions (width 105 mm, height 23 mm, and depth 33 nm). The MinION exhibits real-time sequencing to get up to 48 Gb data from a single flow cell with theoretical max output for 72 hours running system at 400 bases/second. MinION can read the length of nucleic acid in a wide range from short to ultra-long (longest > 4Mb) and can be used for whole genomes/exomes, metagenomics, target sequencing, whole transcriptome (cDNA), smaller transcriptomes (direct RNA), and multiplexing for smaller samples [24]. The newest nanopore R10 series was released in 2020 and exhibited a high accuracy of 99.995% single molecular consensus [25].

B. Nanopore sequecing process

Typically, the nanopore systems work as a biosensor, which consists of certain nanopores located in a resistive membrane and motor enzyme. In the sequencing process, the electric field allows the nucleic acid (DNA/RNA) to translocate through nanopores. The movement of nucleic acid to pass through the nanopore is pulled and controlled by motor proteins. During the translocation, the charge of the nanopore changes, leading to the changes of electrons flow on the resistive membrane. Different bases provide unique changes in the current flow signal which is obtained, recorded, and analyzed by algorithmic programs to identify the base type. Accordingly, an electrical signal is interpreted

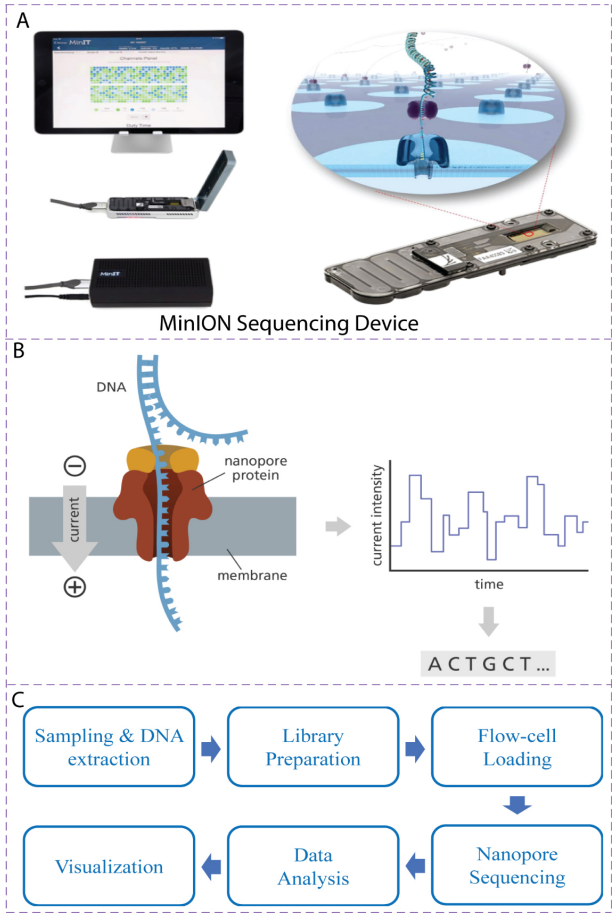

Fig. 2: (A) MinION sequencing device, reproduced with permission from Ref. [26], Copyright 2022 Frontiers; (B) The current signal is interpreted and results in sequencing data, reproduced according to the Ref. [27]; (C) General workflow of ONT sequencing process.

and results in sequencing data (Figure 2B) [27]. The workflows of ONT consist of sampling and DNA extraction, library preparation, loading flow cell, sequencing, data analysis, and visualization (Figure 2C).

III. NANOPORE SEQUENCING TECHNOLOGY IN PATHOGENIC DIAGNOSTICS

The nanopore sequencing method can be applied to different types of sequencing, such as metagenomic sequencing, whole-genome sequencing, and targeted sequencing, because of the diversity of kits for library preparation (Figure 3) [28]. These three sequencing types are effective in the detection of different pathogens, including bacteria, fungi, viruses, and parasites [29–34]. For example, a nanopore-sequencing-based workflow was developed to identify fungal communities in clinical samples of pneumonia patients (Figure 4) [29, 34]. In the workflow, targeted regions that covered partial small subunit ribosomal RNA and large subunit ribosomal RNA, and whole regions of 5.8S RNA, ITS1, and ITS2 were used. The outcomes of the nanopore sequencing method were analysed with reference databases, showing a high accuracy of fungal communities at the genus level of the mock sample and the presence of *Candida albicans* in clinical samples. Other fungal species, such

as *Candida haemulonii*, *Pneumocystis jiroveci*, and *Aspergillus* sp., were also found in clinical samples of lower respiratory tract infection patients [29, 30, 33]. Besides fungi, the nanopore sequencing method was successfully used to trace *Plasmodium falciparum* (causing malaria) with its antimalarial resistance markers [35, 36]. Consequently, an end-to-end molecular surveillance workflow was developed based on the nanopore sequencing method and can be widely utilized in hot spot regions of malaria.

Another effective capacity of the nanopore sequencing technology is that it can identify pathogenic microbial and antimicrobial resistance with the advancement of time-saving and higher efficiency than traditional culture methods. Particularly, nanopore metagenomic sequencing exhibited the ability to clinically diagnose bacterial lower respiratory infection much faster than culture [38]. In a previous study, Charalampous et al. [38] optimized the pilot test, achieving 96.6% sensitivity and 41.7% specificity for pathogen detections, which were then confirmed by quantitative PCR and pathobiont-specific gene analysis, increasing to 100% specificity and sensitivity. Another comparative analysis revealed a rapid pathogenic identification of the nanopore sequencing method, of which the detection time was 2.4 fold and 13 fold lower than those of the MGISEQ-2000 platform (BGI-Tianjin, Tianjin, China) and traditional culture method, respectively [31]. These studies revealed the advantages of the nanopore sequencing method as a rapid and accurate tool for pathogenic detection. Most recently, Chapman et al. [39] have summarized the application of nanopore metagenomic as a developing diagnostic platform in respiratory tract infection. Nanopore sequencing technology can also identify pathogenic microbial and antimicrobial resistance with the advancement of time-saving and higher efficiency than traditional culture methods. As a result, the use of broad-spectrum antibiotic treatment can be reduced and enhance the overall clinical outcomes.

During the COVID-19 pandemic, ONTs have worked as potential tools for clinical sequencing to provide the whole genome information of SARS-Cov-2. The advancement of NGS involves high specificity and the ability to simultaneously identify pathogenic microorganisms such as respiratory viruses, confirming results of other diagnostics methods such as RT-PCR, LAMP, and other biological testing. Whole genome sequencing using the primers and protocol reported by the MinION Mk1C sequencer was adapted and allowed the detection of multiple single-nucleotide variants and assessed the circulation of SARS-CoV-2 VOC Alpha, Beta, Gramma, and Delta [40]. Long-read sequencing of ONT offered optimization of turnaround time, portability, and cost [41]. Bull and coworkers have investigated how ONT sequencing reads can obtain an analysis of single nucleotide variants with high accuracy with a sensitivity of > 99% and precision of > 99% above the minimum coverage depth of 60-folds. However, the authors also revealed that at this period of study, ONT sequencing was not suitable for the accuracy detection of short ideals and variants with low read-count frequencies. These evaluations provided insight into the suitability of ONT sequencing for SARS-CoV-2 genome analysis, especially the pathogenic WGS, allowing wide implications to ensure public health initiatives. Another outbreak, monkeypox virus disease, was rapidly identified using both nanopore sequencing and Illumina platforms [42]. However, the complete genome of the monkeypox virus was assembled using only nanopore sequencing data [43]. Recently, the nanopore sequencing method resulted in a transcriptomic profile of the monkeypox virus and its host which provided critical information to investigate the virus-host interaction and viral infection [44].

Most of the research reported the applications of the nanopore sequencing method on various pathogens of human beings. However, this method was also effective in the pathogenic diagnostics of plants, animals, and environmental samples [26, 45–47]. For example, different phytopathogenic and endophytic fungi such as *Cladosporium*, *Didymosphaeria*, *Paraconiothyrium*, *Penicillium*, *Phoma*, and *Verticillium*

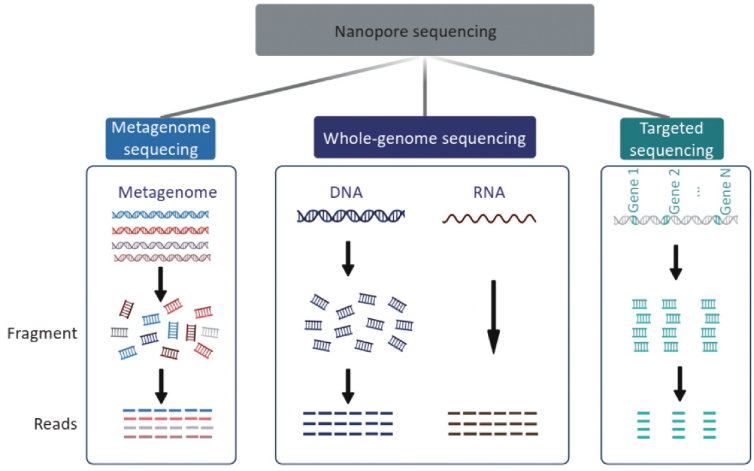

Fig. 3: Capacity of nanopore sequencing technology for different types of sequencing, reproduced with permission from Ref. [37], Copyright 2022 BES.

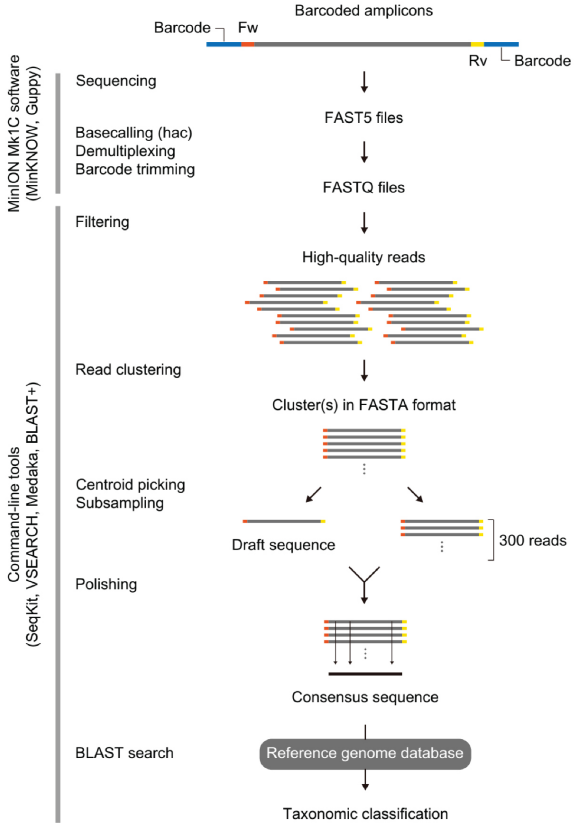

Fig. 4: DNA metabarcoding workflow-based target gene marker using PCR and nanopore sequencing (Fw-forward primer; Rv-reverse primer; hac-high accuracy), reproduced with permission from Ref. [34], Copyright 2023 Springer Nature.

species were detected in the olive tree using ONT sequencing for three DNA loci of ITS, beta-tubulin, and 28S LSU regions [48]. Additionally, a novel all-in-one diagnostic assay based on the nanopore sequencing method was developed for the quarantine of pathogens in plants that was completely done within 13 minutes [49]. The nanopore sequencing method was also effective in virus detection of plum pox virus, cassava mosaic virus, chlorotic necrosis virus, chlorotic stunt virus, and yellow leaf curl virus [26]. Besides plants, the nanopore metagenomic sequencing method could be used for the detection of bacteria and viruses in pigs and cats [50]. The presence of *Mycoplasma bovis* in cattle was also quickly detected with high accuracy using a nanopore sequencing platform [51]. The pathogens are not only found in the host but also the environment. Therefore, the detection of pathogens in different environments such as soil, river, water, and air samples was conducted using ONT [45, 52–55]. The nanopore sequencing method was also successfully applied for the detection of antibiotic resistance genes in microbes [45, 53]. These results revealed the potential, flexibility, and accuracy of the nanopore sequencing method for pathogenic diagnostics.

IV. CONCLUSION AND PERSPECTIVE

This mini-review provided fundamental information about nanopore sequencing technology and its application for pathogenic diagnostics. The nanopore sequencing method exhibited effectiveness for the detection of pathogens in not only human beings but also in other hosts such as plants and animals. Additionally, the nanopore sequencing method was also applied in various research fields such as agriculture (i.e., selective breeding), the food industry (i.e., food safety), and environmental science (i.e., biotic pollutants). The lower accuracy of the nanopore sequencing method compared to other technologies can be resolved with the development of technology in the near future. Currently, due to the diversity of kits for sequencing preparation, portable devices, and effective bioinformatic tools, nanopore sequencing technology has a great capacity for applications that can be applied anywhere, for anything, by anyone. However, two obstacles to the wide-range application of nanopore sequencing are possible genomic databases and specific workflows that enable high accuracy and fast analysis of pathogenic detection. Therefore, new pipelines and suitable genomic databases that are user-friendly and easy to use should be constructed to create multifunctional systems for pathogenic diagnostics.

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