Nanopore-based Metagenomic Sequencing in Respiratory Tract Infection: A Developing Diagnostic Platform

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Disclosures and Conflicts of Interest:

RC, AS, MA, SB report no conflicts of interest or funding related to this work

LJ is a PhD candidate partly funded by Oxford Nanopore Technologies

KEYWORDS:

Nanopore sequencing, Next generation sequencing, Respiratory Tract Infection, Diagnostics, Mycobacteria, Tuberculosis, Antimicrobial Stewardship

ABSTRACT

Respiratory tract infection (RTI) remains a significant cause of morbidity and mortality across the globe. The optimal management of RTI relies upon timely pathogen identification via evaluation of respiratory samples, a process which utilises traditional culture-based methods to identify offending microorganisms. This process can be slow and often prolongs the use of broad-spectrum antimicrobial therapy, whilst also delaying the introduction of targeted therapy as a result. Nanopore sequencing (NPS) of respiratory samples has recently emerged as a potential diagnostic tool in RTI. NPS can identify pathogens and antimicrobial resistance profiles with greater speed and efficiency than traditional culture-based methods; potentially improving antimicrobial stewardship by reducing the use of broad-spectrum antibiotic therapy, as well as improving clinical outcomes. This new technology is becoming more affordable and accessible, with some NPS platforms requiring minimal sample preparation and laboratory infrastructure. However, questions regarding clinical utility and how best to implement NPS technology within RTI diagnostic pathways remain unanswered. In this review, we introduce NPS as a technology and as a diagnostic tool in RTI in various settings, before discussing the advantages and limitations of NPS, and finally what the future might hold for NPS platforms in RTI diagnostics.

INTRODUCTION

Respiratory tract infection (RTI) is a leading cause of mortality globally, as well as a significant cause of morbidity amongst previously healthy adults [1]. Effective treatment of RTI relies upon efficient diagnosis and rapid delivery of appropriate treatment, usually in the form of antimicrobial therapy [2]. Whilst polymerase chain reaction (PCR) technologies have

become mainstream within viral RTI diagnostic panels, a practice highlighted by the coronavirus pandemic, their use within bacterial RTI is extremely limited. Diagnostic strategies within this area still rely heavily on traditional culture methods, which are often slow and sometimes generate ambiguous mixed growth results or discard important results as oral flora [3].

Reducing time to pathogen identification in bacterial RTI represents an important focus of study for two reasons. Firstly, delivering a targeted antibiotic sooner would almost certainly improve clinical outcomes, and secondly it would greatly reduce the use of broad-spectrum antibiotics, attenuating drivers of antimicrobial resistance. Whilst multiplex bacterial PCR improves time to diagnosis in RTI [4], it is third generation sequencing (TGS) techniques such as Nanopore sequencing (NPS) that offer real promise as a new diagnostic tool.

Nanopore technology can sequence metagenomic samples quickly, requiring basic preparation to generate accurate genomic data that permits pathogen identification [5,6]. Multiple studies using Nanopore technology to sequence respiratory samples and identify pathogens [7] have demonstrated that NPS is a feasible option as a diagnostic tool in RTI. In this review, we describe the origins of NPS through to its present use and potential future use as a clinical tool in RTI.

NANOPORE SEQUENCING

Origins & Development

NPS was first described in the 1980s with translocation of single-stranded polynucleotides via an electrically charged α-hemolysin (αHL) pore [8]. Associated studies demonstrated the

potential to characterise base pairs by monitoring bases' differing disruption to ionic current. This led to a patent in 1995 by Church, Deamer, Branton and colleagues [9]. Kasianowicz and colleagues worked to improve understanding and throughput of the Nanopore approach by preventing spontaneous pore gating (pore closure) and by demonstrating the capability to sequence both RNA and DNA directly, with indiscriminate initiation from 3' and 5' ends [8].

Identification of individual bases was demonstrated in 2005, starting with adenine, and a modified αHL was subsequently shown to be capable of distinguishing between the four bases of DNA within homopolymers and heteropolymers [10,11]. These approaches were limited by the reliance on DNA immobilisation within the pore. This was later overcome using an MspA mutant pore, which provided higher accuracy due to greater field disruption [12].

Oxford Nanopore Technologies (ONT) licensed NPS in 2008. This led to the release in 2012 of the MinION, a USB-powered 100g device capable of rapid, high sensitivity sequencing across 2,048 separately embedded pores. The MinION and other ONT sequencing instruments are controlled and complemented by ever advancing software produced by ONT and third parties. In recent years, packages such as EPI2ME, which offers all-in-one workflow management, have simplified the computational side of the workflow (see Figure 1). Other key advances in NPS technology in the last decade include improvement in read identity/accuracy from 60-85% to >99.9%, partially assisted by post-sequencing computational analyses, like consensus calling (Figure 1) [13,14], and detection of DNA methylation [15]. Due to its fundamental role in cancer, methylation detection offers a

unique diagnostics opportunity [16]. These and other developments are allied with the sensitivity to detect mutants in a 1:100 dilution, as demonstrated in oncological biosensing [17].

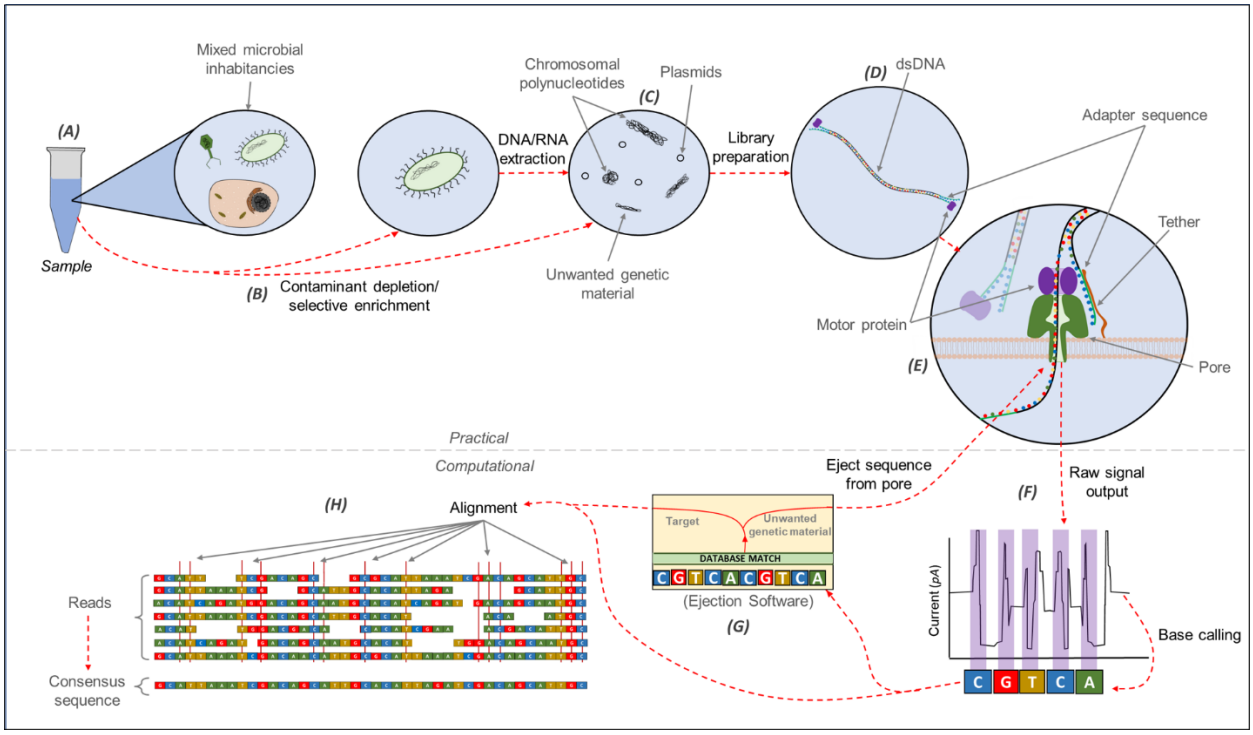

Figure 1: *Simplified workflow for Nanopore sequencing. (A) Samples with varying diversity of constituents can be used. (B) Various isolation protocols can be used, depending on the nature of the desired polynucleotides. (C) The desired genetic material is often refined along with unwanted genetic material, which in a clinical context would be host DNA. Such unwanted genetic material reduces the concentration of the targeted genetic material, weakening the desired signal during sequencing. Depletion protocols are therefore often used to reduce host DNA, enriching microbial signal. (D) During library preparation, adapters tagged with motor protein are ligated onto polynucleotides. (E) Sequencing is initiated by the adapter being guided to an available pore by attachment to a tether. The motor protein then attaches to the pore, where it enzymatically separates the strands into template and complementary strands. The potential difference across the membrane pulls the template strand through the pore, most often releasing the complementary strand, although in 10-20% of cases, the complementary strand is also pulled through the pore to produce a duplex read of both strands. (E) As the polynucleotide passes through the pore there is a disruption in current flow which is unique to each of the four bases, and indeed to modified versions of those bases such as 5-methylcytosine. The output of this*

disruption is the raw signal, which is converted to the sequence of bases in a process known as base calling (F). (G) Some workflows utilise adaptive sampling in which the sequencing output is compared in real time with pre-selected sequences; any strand not matching the pre-selected sequences is ejected from the pore. Adaptive sampling allows enrichment of desired signal, for example signal from microbial DNA versus signal from host DNA. (H) The resulting reads are aligned, and once sufficient reads have been aligned, high confidence consensus sequences can be formed.

NPS Versus Other Third Generation Sequencing Techniques

TGS methods can produce long reads, facilitating genome sequence assembly and allowing sequencing of complete transcripts. Two TGS modalities exist: single-molecule-real-time (SMRT) sequencing from Pacific Biosciences (PacBio), and NPS from ONT, each including several devices (Table 1) and associated bioinformatics tools. MinION (ONT) is the cheapest TGS device; it is portable and has relatively low infrastructure requirements (Table 1). Unlike previous sequencing methods, particularly those based on PCR which require some level of insight for primer selection/design, TGS methods can be agnostic; by avoiding amplification, TGS methods can produce results which accurately reflect the diversity and proportions of microbial populations in a given sample [18,4]. This leaves the challenge of differentiating between microbiota and pathogen.

ONT and PacBio platforms are capable of RNA sequencing, typically requiring indirect sequencing via translation to cDNA, often with an amplification step [19]. Nanopore devices are, uniquely, also capable of direct RNA sequencing, providing a theoretical advantage in clinical diagnostics where rapid detection is often vital. In the respiratory tract, for example, where viral disease is prominent, several common viruses are RNA-based, including SARS-CoV-2, respiratory syncytial virus, and influenza A.

TABLE 1						
Platform	**Long-read modality** (Upfront cost)	**Consumable cells** (Upfront cost)	**Consensus sequence accuracy**	**Run duration**	**Maximum throughput** (bases, b)	**Infrastructure requirements**
PacBio *(Cheapest setup)*	Sequel (Price not Listed)	SMRT Cell 1M (Price not listed)	>99%	*ca.* 20 hours per cell	20 Gb per cell	• Ethernet-based (wired) high speed internet access. • Sequencing Kit. • An appropriate flow cell(s). • Server and cloud arrangements.
PacBio *(High-end setup)*	Revio *ca.* $779,000 (≈£629,221)	Nanofabricated SMRT Cells (Price not listed)	>99.95%	24 hours	360 Gb per day	• Ethernet-based (wired) high speed internet access. • Sequencing Kit. • Appropriate flow cell(s). • Server and cloud arrangements.
ONT *(Cheapest setup)*	MinION £900 (Basic starter pack included)	R10.4.1 $810 for 1 ($810 down to $430 each for bulk purchases)	>99%	*ca.* 72 hours per cell	≤50 Gb	• Computer/Laptop with a graphics processing unit (GPU) is recommended. • Sequencing Kit. • An appropriate flow cell.
ONT	GridION £44,960 (Extensive starter pack included)	R10.4.1 $810 for 1 ($810 down to $430 each for bulk purchases)	>99%	*ca.* 72 hours per cell	Up to 250 Gb (50 Gb per flow cell)	• Method of transporting output data (USB drive), or an ethernet-based server connection. • Sequencing Kit. • Appropriate flow cell(s).
ONT	PromethION 2 Solo (P2 Solo) £9,411 (Starter pack included)	R10.4.1 $5,040 for 4 ($1260 down to $540 each for bulk purchases)	>99%	*ca.* 72 hours per cell	580 Gb (290 Gb per flow cell)	• Computer, or a GridION. • Sequencing Kit. • Appropriate flow cell(s).
ONT	PromethION48 £279,071 (Extensive starter pack included)	R10.4.1 $5,040 for 4 ($1260 down to $540 each for bulk purchases)	>99%	*ca.* 72 hours per cell	≤14 Tb (290 Gb per flow cell)	• Method of transporting output data (USB drive), or an ethernet-based server connection. • Sequencing Kit. • Appropriate flow cell(s).

Table 1: Comparison of ONT and PacBio third generation sequencing platforms. The table shows example setups and the associated potential for these setups as claimed by the respective companies on their websites.

Only long read-capable systems were included (e.g., PacBio's ''Onso'' system is excluded). Reliable pricing could not be found for all equipment [20,21,22].

NANOPORE SEQUENCING IN RTI

The implementation of metagenomics in clinical settings was Initially hindered by capital and maintenance costs, requirement for highly skilled staff, and uncompetitive turnaround times compared to traditional culture-based methods. More recent sequencing techniques, however, offer much reduced turnaround times, reduced resource and skill requirements, and lower capital and maintenance costs. Nanopore-based sequencing platforms are already being investigated as diagnostic tools in RTI, with promising results reported in a range of clinical settings [7].

Viral Pathogens

Viral pathogens are common drivers of acute RTI in both adults and children. PCR-based methods are already commonplace in diagnostic panels; examples include Influenza A and SARS-CoV-2 diagnostics, where PCR allows rapid and accurate detection of infection [23,24]. NPS methods have been shown to be as accurate as PCR-based methods with regards to common viral RTI, with the crucial advantage of generating real-time data whilst also being more portable and requiring less laboratory infrastructure [25,26]. NPS methods, moreover, permit whole viral genome sequencing which enables large-scale epidemiological surveillance, crucial in viruses that have the potential to mutate rapidly in key genomic locations [27].

Bacterial Pathogens

Historically, bacterial RTI diagnostics in clinical settings have been limited, and they continue to be limited, to traditional culture methods, utilising broncho-alveolar lavage (BAL) or sputum samples as substrate [28]. UK guidelines advise a strategy of commencing broad-spectrum antibiotic therapy in patients with RTI, adjusting to more focused therapy once culture results are available [29]. On average, culture results take 48-72 hours to be made available to physicians. As a result, patients remain on broad spectrum antibiotics for extended periods, potentially contributing to the development of antimicrobial resistance. Likewise, the delay in targeted antibiotic therapy negatively affects patient outcomes [30,31].

NPS has been shown to be an accurate and cost-effective method of diagnosing bacterial RTIs in various clinical settings [7,32,33]. Notably, a turnaround time of under 6 hours (sample received to pathogen identification time) has been reported for the diagnosis of bacterial RTI [34], including data on antibiotic resistance profiles [35]. Such speed would allow patients to receive targeted therapy sooner and would permit sharply reduced use of broad-spectrum antibiotics, leading to better patient outcomes and improved antimicrobial stewardship.

Mycobacterial Pathogens & Pulmonary Tuberculosis

Pulmonary tuberculosis represents a huge burden on global respiratory health and is often complicated by patterns of multi-drug resistance [36,37]. PCR-based diagnostic methods are in use for suspected cases of pulmonary tuberculosis [38], but traditional culture of BAL or sputum sample remains the gold standard for diagnosis [39]. Culture results can take 4-6

weeks to be confirmed, representing a significant area of diagnostic delay [40]. Non-tuberculous mycobacteria pulmonary disease (NTM-PD), moreover, is rising in incidence globally [41]. NTM-PD is driven by a multitude of mycobacterial species, and is again diagnosed by traditional mycobacterial culture, which often takes 4-6 weeks to process and obtain results [42]. PCR-based methods are less common here and are often targeted at *Mycobacterium tuberculosis* rather than mycobacterial species more broadly [43]. NTM-PD therapy may therefore be delayed until confirmatory culture results are received, with potential negative effects on patient outcomes. NPS is effective in detecting mycobacterial species (including *Mycobacterium tuberculosis*) in respiratory samples, with accuracy and turnaround times comparable to those mentioned above for bacterial RTI [44,45].

ADVANTAGES AND LIMITATIONS OF NANOPORE SEQUENCING

Read Length

NPS can output ultra-long reads, as previously emphasised. For example, consistent read lengths on the order of 880kb have been reported in non-clinical specimens [15]. Ultra-long reads can, however, run into error rate issues when translocation speed slows in the later stages of runs. This issue can now be resolved using 'refuelling buffer' [46], reflecting ongoing innovation that has made NPS more reliable and capable, regardless of target sequence, qualities that are vital for robust diagnostic tools. Clinically, long read lengths can allow for whole genome sequencing of smaller (mainly viral) genomes, and thus potentially produce highly specific diagnostic tests and outbreak surveillance platforms. Moreover, long read lengths can potentially aid in the investigation of antimicrobial resistance by sequencing (in a single read) complex areas of pathogen genomes where these genes may be found.

Time to Antimicrobial Resistance Data

Treatment efficacy in infectious disease relies heavily on the speed with which appropriate treatment is identified. Antibiotic Susceptibility Tests (ASTs) are currently the gold standard as they identify phenotypical resistance. A proof-of-principle study conducted on *Klebsiella pneumoniae*, testing NPS's ability to replace current ASTs, found 80-100% concurrence (averaging 92%) between ASTs and NPS results, when NPS is paired with computational assembly. Whilst ASTs can take >72 hours, results from NPS were available after 38 hours [47]. NPS time to result can be reduced using more specialised approaches such as saponin-based depletion of host DNA. In the case of *E. coli*, for example, the resistance genes bla_{TEM}, *sulf1* and *dfrA17* were undetected after two hours of sequencing untreated samples, besides one alignment of *sulf1*. In saponin-depleted samples, in contrast, all three of these resistance genes were detected within the first 20 minutes [7]. Similar improvements can be achieved via other methods. An enzyme-based host depletion protocol in combination with adaptive sequencing (Figure 1), for example, showed a 113.41-fold increase in the median of microbial reads [48]. These results demonstrate that NPS, when paired with efficient host DNA depletion, is capable of rapid and broad AMR detection. The primary constraint is currently translation of genotype into phenotypical resistance.

Accuracy & Sensitivity

NPS is sometimes criticised for relatively low raw output accuracy, with base calling errors previously encompassing 5-25% of a given sequence [8]. Whilst NPS accuracy has been relatively low amongst TGS methods, it has improved and is still improving through changes to the pore protein and sequencing chemistry, alongside developments in software. In a

recent iteration, ONT claimed 99.6% raw accuracy using flow cell R10.4.1 and ''super accuracy'' base calling [49]. In practice, R9.4.1 achieved 95.5-98% accuracy in a diverse activated sludge microbiome, when paired with Guppy V6.0.0 base-caller [50]. NPS paired with consensus calling has achieved 100% accuracy for SARS-CoV-2 when compared with Sanger sequencing, the current gold standard for clinical research [51].

NPS has already been shown in some cases to be more sensitive than traditional diagnostics, and the benefits of agnostic metagenomics in identification of culture-negative pathogens have been highlighted [52]. More recently, the sensitivity and specificity of NPS diagnoses, assessed using patient samples, were found to be 94.5% and 31.8%, respectively, across fungal and bacterial infections; NPS was found to be 56.7% more accurate with regard to true positives than culture methods, while retaining inaccuracy in true negatives [53]. For viral pathogens, the sensitivity and specificity are much higher, with 99.1% and 99.6% respectively for SARS-CoV-2 [54], and 100% sensitivity for avian Influenza A [55].

Combining NPS With Other Methods

As previously mentioned, NPS has several unique benefits. In addition, numerous methods exist to enhance its suitability for clinical use, though these can introduce other issues. Using a Cas9/sgRNA complex to protect desired sequences from exonucleases, for example, provides an effective 'selective enrichment' alternative to depletion protocols. This approach is hindered, however, by a restriction to short reads and a requirement for knowledge of the target(s) [56].

Using the MinION to sequence amplicons produced by a PCR assay has provided high confidence positive results with bacterial pathogens within the first ten minutes of

sequencing. This involved a diagnostic pipeline capable of processing 45 samples across 12 hours; though this was hindered, however, by excessive false positives/negatives [57]. Combining high-throughput NGS platforms and NPS has found success in pathogen and AMR detection; this method took 212 hours (from sample to results), 12 hours of which were the NPS stage [58]. Such methods have shown greater precision and accuracy than culture methods but at the cost of a considerably longer time requirement with consequent increased demand for resources and skilled staff, compared to methods using only NPS.

Sample Requirements & Analysis

An important limitation of NPS is the requirement for relatively large amounts of sample, currently up to a few micrograms of DNA and hundreds of nanograms of RNA. Considering that biomedical investigations often rely on a limited amount of genetic material, reducing the amount of sample needed for NPS would promote uptake. More user-friendly bioinformatics platforms and sufficient cloud storage would further promote the application of NPS in clinical settings.

ADDITIONAL DIAGNOSTIC ROLES

Outbreak Surveillance

NPS can be used for real-time and field genomic surveillance of potential new infectious diseases. These epidemiological and phylogenetic investigations can result in the timely identification of potential diagnostic targets and treatments, as well as monitoring the evolution and transmission rate of the new infection. For example, NPS was used to conduct genomic surveillance of the yellow fever virus [59], Zika virus [60] and dengue virus [61] worldwide. A Salmonella outbreak in an American hospital was identified using NPS with all

positive cases reported within two hours [62]. Real-time genomic surveillance using NPS was carried out in Guinea for the ongoing Ebola virus outbreak [63]. Real-time genomic surveillance has been more recently applied to pathogens with large genomes including bacteria such as *K. pneumoniae* and *N. meningitidis* [64,65], fungal pathogens such as *Candida auris* [66], and large viruses such as Lassa fever virus [67], Zika virus [60], Venezuelan equine encephalitis [68] and SARS-CoV-2 coronavirus [51].

Other Infectious Diseases

Thanks to its real-time sequencing capability, NPS has been used for rapid pathogen detection in prosthetic joints [69], bacterial meningitis [70], infective endocarditis [52] and pneumonia [71]. In six retrospective meningitis cases, NPS detected pathogenic bacteria in only ten minutes, corroborating the idea that NPS can permit early administration of antibiotics following the timely identification of pathogenic bacteria [70]. It is worth mentioning that NPS can additionally be used for the investigation of antimicrobial/antibiotic resistance in different microbes. For instance, NPS was used to detect 51 acquired resistance genes from clinical urine samples with no need for culture [5]. More recently, NPS was used to identify resistance genes to colistin in 12,052 strains of Salmonella [72]. Thanks to the ability to perform longer reads, NPS is a robust technology for the identification of virulent strains and species, ultimately providing a reliable estimate of microbiome composition [73,74].

CONCLUSION

NPS has advanced significantly as a diagnostic platform over the last decade and has now reached the tipping point of becoming a feasible addition to clinical diagnostic panels within hospitals and medical outreach centres. The advantages of NPS are multitude and provide

clear clinical benefit (reduced time to pathogen identification, broader search panels), as well as public health benefits with regards to reducing inappropriate antimicrobial use and improved outbreak surveillance. The associated cost, training and infrastructure required to establish NPS platforms have, to date, limited its widespread adoption, but these aspects continue to improve and are the focus of ongoing research within the field. With this in mind, NPS technology should be initially instituted in clinical areas where it can be most effective and confers the largest benefit to patients. RTI is an obvious target due to the accessibility of testable specimens, large clinical burden and high transmissibility of disease. Further translational research and large-scale trials are needed to test the utility of NPS in clinical microbiology laboratories.

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