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Review Article

Next-generation sequencing applications in clinical bacteriology

Yair Motro[a], Jacob Moran-Gilad[a,b,c,*]

[a] Department of Health System Management, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel
[b] Public Health Services, Ministry of Health, Jerusalem, Israel
[c] ESCMID Study Group for Genomic and Molecular Diagnostics (ESGMD), Basel, Switzerland

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ABSTRACT

With the rapid advances in next generation sequencing (NGS) technologies, clinical and public health microbiology laboratories are increasingly adopting NGS technology in their workflows into their existing diagnostic cycles. In this bacteriology focused review, we review aspects and considerations for applying NGS in the clinical microbiology settings, and highlight the impact of such implementation on the analytical and post-analytical stages of diagnosis

1. Introduction

Clinical and public health microbiology laboratories help to lessen the burden of infectious disease by detecting and characterizing pathogens in infected patients or those pathogens circulating in the community. In this scenario, implementation of next generation sequencing (see Heather and Chain [1] for an encompassing review of NGS technologies) can potentially assist in clinical and public health decisions by determining the causative agent of infectious disease and/or the epidemiology and evolution of various infecting pathogens in the hospital or community settings [2]. With its multitude of benefits, NGS is becoming the gold standard in bacteriology, however since it is not yet fully accessible (particularly in low resource settings), currently NGS is mainly used at a level of reference microbiology rather than routine [3].

Traditionally, the clinical diagnostic cycle consists of three phases, namely, a pre-analytical phase (including, patient visit, examination and provisional diagnosis, collection of sample, identity, requisition, transport, records), an analytical phase (including, macroscopy, microscopy, culture, biochemical identification, serology, molecular analysis), and post-analytical phase (including, reporting of identification and antibiotic susceptibility testing, clinical interpretation of the results and patient treatment). With the rapid advances in NGS technologies and capabilities, clinical microbiologists are recognising that the influence of NGS on the diagnostic cycle will be in the scale of a “disruptive technology”, potentially reducing the time from diagnosis to clinical treatment, while also reducing the requirement for wet laboratory-based analyses performed in tandem. In addition, a NGS-based analytical phase will provide the opportunity to apply a broad repertoire of tools, including subtyping, resistome and virulome mapping, phenotypic inference, detection of new variants and toxins, among others [4]. This review will focus on the application of NGS in the clinical microbiology context, with emphasis on the potential role of NGS in the analytical and post-analytical phases of the clinical diagnostic cycle. It should be noted that this review focuses on bacteriology, though the importance of NGS in virology and other fields is of no lesser extent. So too, the NGS technology of focus in this review will be on the currently more widely used second generation sequencing [1].

2. The NGS workflow

Generally, the clinical laboratory workflow may be divided into several stages, namely, pathogen detection, identification, drug susceptibility, epidemiological typing [4], and detection of toxins and virulence factors that have clinical or prognostic implications. Note that bacterial and fungal isolates are detected through these steps, but virus detection and characterisation mainly relies on PCR-based assays. Also, each step involves a range of specialised tests that must be performed individually on each isolated organism [4].

There are several common steps that are shared among the majority of NGS methods, with the exception of single-molecule real-time NGS. A typical NGS workflow in a clinical laboratory includes: sample collection and preparation, nucleic acid extraction, NGS library preparation, sequencing, data analysis, and data storage [4,5].

* Corresponding author at: Dept. of Health Systems Management, Faculty of Health Sciences, Ben-Gurion University of the Negev POB 653, Beer-Sheva, 8410501, Israel.
E-mail address: giladko@post.bgu.ac.il (J. Moran-Gilad).

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2.1. Clinical sample

The clinical sample (for example, swab, sputum, stool, urine, or tissue, that contains the nucleic acid (DNA/RNA) of interest) is stored and transported to the clinical microbiology laboratory. The clinical specimen type depends on the patient's clinical syndrome, where ideally sample are collected during disease progression. Notably, the eventual NGS method that will be required (for example, whole genome sequencing (WGS) or metagenomics) will also influence the type of treatment of the sample (i.e. sample culturing or direct sample extraction).

2.2. Nucleic acids preparation

Nucleic acids can be prepared from clinical samples by using a variety of methodologies, some of which are dependent on the NGS system being used. Correct method selection is essential for a successful result, thereby lessening the introduction of biases and false negatives. Notably, the DNA quantity and quality required for NGS are higher than standard molecular assays and manual or automated platforms used for routine extraction for molecular diagnostics are not always fit for NGS. From our experience, extraction for NGS commonly requires tailoring or calibrating methods, at least for certain bacterial species, since a 'one size fits all' method is not readily applicable.

2.3. Nucleic acids sequencing

In general, two main NGS platform methods are currently used, namely short read platforms (including Illumina and Ion Torrent) and long reads platforms, including single molecule real time sequencing (Pacific Biosciences) and nanopore (Oxford Nanopore) sequencing (see Levy and Myers [6] and Kwong et al. [7] for detailed reviews and comparisons of these platforms). The input nucleic acid (for example, genomic DNA, reverse-transcribed RNA or cDNA, immunoprecipitated DNA) is firstly fragmented by methods such as sonication, nebulisation, or enzymatic digestions [8]. The fragments are then ligated to platform-specific oligonucleotide adapters to create a library of overlapping sequences, which is then hybridised to beads or a flow cell, followed by clonal amplification, such as emulsion PCR or bridge amplification (note that not all platforms require the clonal amplification phase or preparation of a DNA library). Enrichment procedures can also be completed at this stage to help select for a specific type of DNA if an organism is suspected. Of note, Becker et al. [9] compared six bacterial DNA extraction kits for a subsequent MiSeq sequencing run of a clinical *Klebsiella pneumoniae* sample, and noted that the choice of extraction kit had little effect on sequencing read quality and sequencing coverage, rather the extraction costs, extraction time, robustness and reproducibility as well as the potential for automation are the main factors for selecting a fitting extraction procedure.

2.4. Sequence data analysis

Depending upon the NGS platform, the clonally amplified templates are sequenced by various chemistries (such as pyrosequencing, reversible dye terminators, oligonucleotide probe ligation, and phospholinked fluorescent nucleotides), and following quality control and assurance of the sequence data, analysis is preformed to determine the composition of the DNA sequences for pathogen identification.

2.5. Data release and clinical report

The final stages of the NGS workflow are data release and dissemination of a clinically actionable report. Appropriate NGS analysis files should be stored or archived on- or off-site with patient privacy/confidentiality upheld [8], allowing for future re-examination upon request.

3. Impact of the NGS workflow on clinical microbiology

3.1. Less technical laboratory involvement

With the progression of the sample in the clinical laboratory workflow, the involvement of the hands-on technician at each successive step is required, particularly where additional challenges are posed by particular organisms, some of which may be of critical public health importance. For example, *Mycobacterium tuberculosis* complex bacteria are extremely slow growing, taking weeks to 1–2 months to achieve susceptibility results, thus delaying appropriate treatment and potentially negatively impacting the patient outcome [10]. Furthermore, many aetiological agents, such as *Borrelia burgdorferi* (causative agent of Lyme disease), *Bartonella* species, *Mycobacterium leprae*, and HIV elude conventional testing altogether [11] (for an example, see the latest developments in HIV clinical treatment and surveillance using NGS reported by Metzner [12] and Berg et al. [13]). Here NGS technologies may be applied for the identification of unculturable or difficult-to-culture microorganisms, including fastidious bacteria, anaerobes, and possible bioterrorism agents [10,14].

3.2. Reduction in patient diagnosis time

In addition, in the case of patient care, where time is critical, rapid infection identification and diagnosis is imperative. For example, in the case of encephalitis up to 60% of acute cases go undiagnosed, possibly due to a lack of assays that can test for the more than 100 aetiological agents that may cause the disease [11] as well as non-infectious aetiologies. In turn, prior to knowing the infecting pathogen, clinicians commonly are forced to educated guesses regarding the therapy, consequently leading to delays and a risk of ineffective treatment and further spread of infection. Furthermore, administration of broad-spectrum empirical therapy may be opted, potentially causing "collateral damage" by eliminating helpful gut microbiota while also accelerating antimicrobial resistance development. Here too NGS holds significant promise, offering potential faster and more reliable detection methods.

3.3. Wider diagnostics repertoire

NGS offers the capability of identifying a variety of organisms—bacterium, virus, fungus, yeast, or parasite, as opposed to a variety of individual tests traditionally required to identify a pathogen [4]. Unbiased or agnostic NGS amplifies all nucleic acids present in a clinical sample, including both host and microbes, without requiring primers for targeted amplification, and can potentially generate microbial sequence data for real-time patient management [4], providing great potential to impact patient care by assisting the customization of patient treatment, while in turn reducing the usage of ineffective drugs and selective pressure for resistance development [15].

3.4. Further benefits from the NGS workflow

In an encompassing review [16], five main areas of benefit for clinical microbiologists from the applications of NGS were identified, including (a) clinical identification from primary samples or a pure culture [17], (b) infection control actions [18], (c) antimicrobial stewardship [19], (d) outbreak investigation in community and hospital settings to guide measures for containment [20], and (e) pathogen discovery [11]. Furthermore, in contrast to other microbial pathogen identification techniques, NGS metagenomics is not restricted to known organismal sequences, thus allowing for comprehensive pathogen detection without *a priori* knowledge of the target organism [21]. Additional NGS benefits include organism differentiation, novel organism discovery, virulence factors and resistance markers elucidation, host response characteristics to the offending microbe and administered