



## Early View

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### **Whole Genome Sequencing of *Mycobacterium tuberculosis***

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## CLEAN COPY OF THE AMENDED MANUSCRIPT

Next Generation Sequencing (NGS) technologies using massively parallel processing to interrogate pathogen genomes in days are revolutionizing the clinical microbiology practice [1]. Whole genome sequencing (WGS) offers unprecedented resolution for genotyping, outbreak investigation and determination of known sequence variants involved in antimicrobial resistance (AMR), which deep sequencing of selected genomic regions (targeted NGS, tNGS) can further illuminate. WGS-based approach has been proposed for surveillance of bacterial pathogens included in the "priority list" by the World Health Organization (WHO) [2]. At present, proof-of-principle and validation studies have been conducted for WGS from culture samples of *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Acinetobacter* spp., *Neisseria gonorrhoeae* and *Clostridium difficile* included in this list, in addition to the globally established priority group *Mycobacteria* spp. (including *Mycobacterium tuberculosis*, MTB) [3].

### Sequencing technologies

For years, the clinical microbiology laboratories relied on the use of conventional phenotypic and genotypic methods for species identification, detection of antibiotic resistance, and for studying transmission of bacteria responsible of hospital- or community-acquired infections that are useful, but have long turnaround times (TAT), require specific infrastructure, and have low discriminatory power to distinguish highly genetically-related strains [4]. Moreover, technological constraints mean that only a few genomic loci can be simultaneously investigated [4]. Sanger DNA sequencing, developed in the 1970s, enabled the first gene and genome sequences but remained limited in its application due to the complexity and high sequencing costs when applied to extended genomic regions [5]. The sequencing output increased tremendously with the advent of pyrosequencing, and then of NGS methods in the mid-2000s when costs plummeted [6]. The NGS platforms generate sequence of unique DNA fragments known as 'reads' at the end of the sequencing reaction either, which can either be short (50-400 base-pairs in length) or long (1-100 kilo-bases). A single NGS run can produce billions of sequence reads [5]. The workflow is similar for all the technologies: extraction of high molecular weight DNA; preparation of libraries (i.e. collection of DNA fragments) through DNA fragmentation (enzymatic or mechanical), barcoding and PCR amplification; clustering (clonal amplification of single DNA fragments); automated single- or paired-end (i.e. the sequencer reads both ends of a DNA fragment) sequencing [5]. Among the major players in the field, Illumina Inc. (San Diego, CA, USA) uses the sequencing by synthesis (bridge PCR) chemistry and offers a wide range of "benchtop" (MiniSeq, MiSeq) or high-throughput (NextSeq 500, HiSeq 2500, Novaseq) solutions allowing higher output at lower costs for generation of high-quality short reads. Thermo Fisher Scientific Inc. (Waltham, MA, USA) "benchtop" (PGM, S5) and high-throughput (Ion Proton) platforms are based on sequencing by synthesis-semiconductor (emulsion PCR) chemistry generating lower throughput but longer reads, and in a shorter time compared to Illumina, thus being well suited for targeted solutions, but with higher error rates in homopolymers [5]. The so-called third-generation sequencing platforms (Pacific Biosciences of California Inc., Menlo Park, CA, USA: RSII, Sequel; Oxford Nanopore Technologies Limited, Oxford, UK: MinION) take advantage of single-molecule real-time chemistry to generate long reads. These lend themselves to *de novo* assembly, which doesn't require a reference genome, and the sequencing of longer repetitive regions which short-reads cannot span. Throughput and quality are however lower compared to instruments generating shorter reads [4, 5]. A detailed description of the main features and costs of different NGS technologies available for use in microbiology field is reported in recent reviews [3-5, 7, 8]. The wide range of instruments marketed by NGS companies allows the user to adopt the most effective technology in the microbiology laboratory according to the purposes (routine, epidemiological and drug resistance surveillance, research), cost considerations (staff, capital investment, infrastructure, software-hardware), workload (facility, multi-disease platform, number of cases), and local availability.

### Role of sequencing in tuberculosis

Drug resistant tuberculosis (TB) is considered a main cause of global morbidity and mortality related to AMR [9]. Multidrug (MDR) and extensively drug-resistant (XDR) forms are hampering TB control and clinical management, with treatment success of 54% and 30% for MDR- and XDR-TB, respectively, (compared to 83% of susceptible forms) [9]. Phenotypic testing is the standard for drug susceptibility testing (DST) despite its challenges that are felt most acutely in resource-limited settings. These include the slow growth rate of MTB, the high-level biosafety infrastructure required, the poor reproducibility and some uncertainties around the proposed critical concentrations for some drugs [10]. As MTB resistance emerges from changes in the genome such as single nucleotide polymorphisms (SNPs), insertions and deletions in genes coding for drug targets or involved in drug metabolic pathways, or from efflux pump upregulation, methods for detecting genome variations represent a breakthrough for the rapid, simple and standardized management of DR-TB cases [10]. There are a number of genotypic tests recommended by WHO for diagnosis of MDR- and XDR-TB, including cartridge-based nucleic acid amplification tests and line probe assays that can be implemented in peripheral TB laboratories [11]. However, these tools target only the “hot-spot” regions of few genes to detect resistance to a restricted number of drugs, and do not always report the exact nucleotide change upon which a prediction of phenotypic resistance is based.

All-in-one solutions are needed to guide individualized clinical decisions for the most complicated resistant cases, at least at a reference laboratory level. NGS is the leading candidate technology in this regard [11]. The absence of integrative vectors and low mutation rate make the MTB genome well suited for sequencing. The one technical challenge is the presence of repetitive and hard-to-sequence regions with high GC content. These require sufficient genome-wide sequencing depth to sequence more accurately, which has cost implications [10].

Multiple studies have used WGS to investigate TB resistance prediction, transmission dynamics and the population structure of MTB complex [10, 12, 13]. In some settings genome sequencing is already being used for the surveillance of disease transmission and the emergence of drug resistance in a population, as well as for resistance prediction for first and second line drugs [14-17]. Further work is being done on the discovery of targets involved in phenotypic resistance and the large-scale validation of resistance-conferring mutations [18]; on taxonomy [19]; on characterizing hetero-resistance and mixed infections; and on virulence and pathogenesis [20]. Feasibility studies to evaluate the introduction of WGS into diagnostic algorithms of routine microbiology laboratories have been conducted in low-burden settings [21-23]. In such contexts the rapid identification of DR-TB and transmission events could help achieve TB elimination [24, 25]. Such feasibility studies have largely used Illumina technology, demonstrating how the integration of WGS from culture into the routine diagnostic workflow achieves accurate and timely reports, available several days earlier than phenotypic DST, at no greater costs.

The on-going need of culturing poses a challenge to the full implementation of NGS as an effective alternative to conventional methods (e.g. MTB/RIF Xpert, Cepheid), particularly in resource-limited settings. Efforts are thus being made to develop protocols for WGS directly from clinical specimens. Such procedures include differential lysis steps, TB enrichment and automated DNA purification [26, 27]. These methods remain expensive at present, and challenged by the low starting TB material and contamination of other genetic material (human and oral flora). Targeted approaches taking advantage of the selective amplification of phylogenetic and DR-related regions may represent a suitable alternative for direct sequencing [27] (Figure).

A population-based surveillance study has been conducted in seven highly endemic countries with support of national and supranational reference laboratories, demonstrating that genetic sequencing targeting well-validated mutations [18] represents a powerful tool to determine/monitor resistance trends to 1<sup>st</sup> and 2<sup>nd</sup> line drugs as a replacement for phenotypic tests that perform sub-optimally in such resource-limited settings [17]. Similarly, WGS is now proposed as the standard to detect transmission chains in real-time and guide public health interventions at the highest resolution compared to traditional molecular methods, through either SNP-based (higher resolution) or core genome multilocus sequence typing (slightly less resolution) approaches [16].

### **Sequencing implementation**

Implementing NGS in TB clinical laboratories is complex and involves strategic planning, procurement, budgeting, sample referral systems, standard operating procedures (SOP), quality assurance, data management (storage, analysis, interpretation and reporting), and human resource (staff and training) considerations. Scale-up of sequencing laboratories requires adequate infrastructure: areas for sample preparation (DNA extraction from clinical isolates/specimens considering related biosafety); a molecular biology environment (pre-, post-PCR; space for NGS instrument); power supply; favourable environment conditions (e.g. controlled temperature, humidity, vibration); network and internet connections; and computing capacity. Equipment and reagents required are platform-specific: it's therefore essential to ensure availability of local distributors and prompt technical support. Several solutions are available for extraction and purification of genomic DNA, including commercially available (para)magnetic- and column-based systems, chemical procedures (e.g. CTAB/NaCl protocol), as well as devices for assessment of quantity/quality of the extracted DNA samples (fluorometer, spectrophotometer) [8]. Library preparation and sequencing reactions are performed according to the

instructions provided by NGS manufacturers. Given the large amount of data generated by NGS, staff with a bioinformatics background are required to handle output data for storage and to run analytic pipelines. Large-scale validation of bioinformatic outputs is still needed and international consortia are putting huge effort in the standardization of post-sequencing analysis processes for the reliable interpretation of DR-TB profiles and epidemiological links. User-friendly solutions have been developed to avoid the need of specific bioinformatics skills that are both freely- [28] or commercially-available. As sequencing data require privacy-secured and long-lasting backup for next use, server- or cloud-based solutions are available for users. Staff require adequate and continuous training and mentoring on wet procedures and on post-sequencing processing.

#### Next steps

To fully implement NGS into routine workflows, methods need to enter validation and certification programmes. Performance, accuracy and reproducibility, quality control steps, quality thresholds (e.g. on the depth/breadth of genome coverage), use of standards and development of SOPs, impact on TAT and clinical management, must all be assessed or evaluated [1, 7]. Microbiology laboratories introducing these technologies will undergo external proficiency testing programmes that are already implemented in TB for molecular testing. Simple but comprehensive clinical reports are crucial to help clinicians arrive at the best decisions in the management of TB cases. Although there is a huge amount of data generated by NGS, our knowledge as to all its meaning remains incomplete, and development of how best to report on this data in the meantime remains ongoing [29]. A report should at least give information on sequencing quality, identification of mutations to infer genotyping and DR profiles, provide details on the exact nucleotide changes and standardized prediction of resistance levels (ideally, based on a literature review of MIC data). WGS and targeted NGS approaches promise to become the future standard for DST and epidemiological investigation in TB, and for other high-priority bacterial pathogens [3, 30]. Additional work is needed to address the feasibility of WGS from clinical specimens, to standardize and automate the laboratory procedures and post-sequencing analyses, and to implement the NGS platforms in low-resource, high-burden settings.

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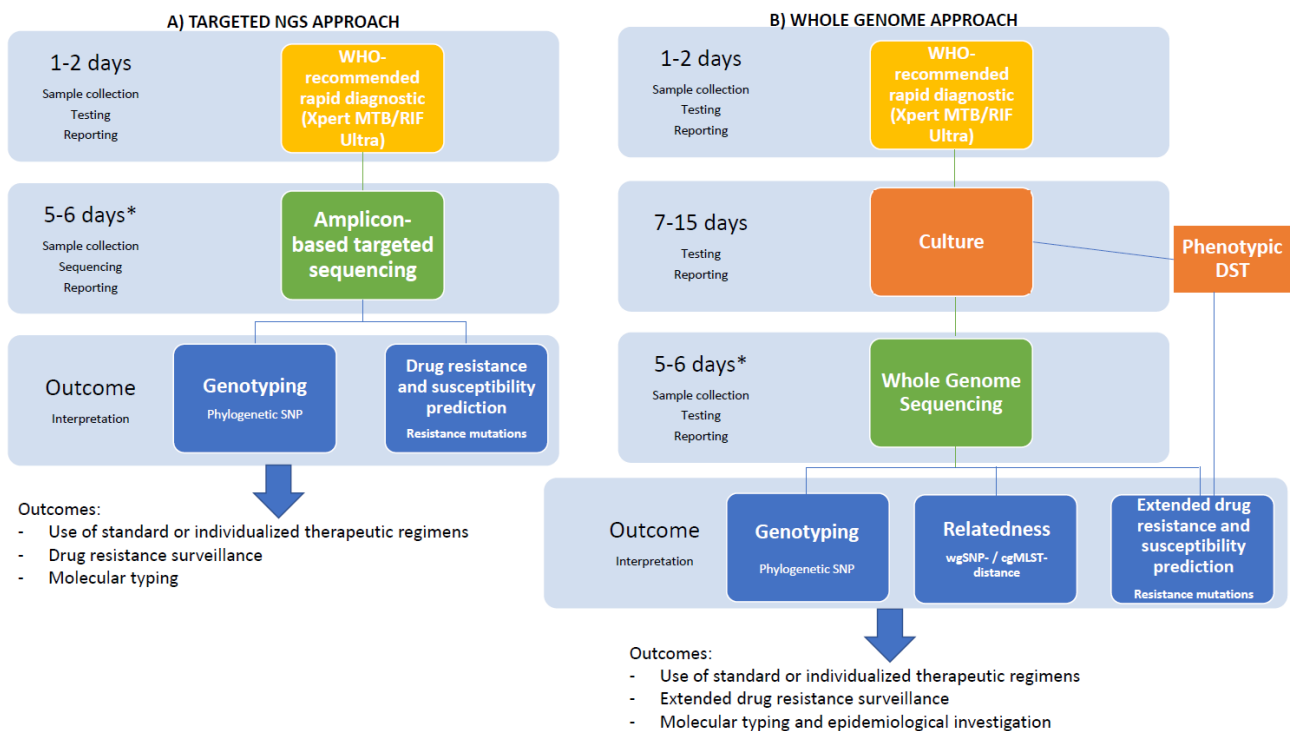
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Figure: “Whole Genome Sequencing of *Mycobacterium tuberculosis*”

Two possible NGS-based scenarios and turnaround times to guide treatment decisions are presented. Modified GLI model TB diagnostic algorithms (Global Laboratory Initiative, March 2017) are outlined here and complemented with NGS approach. A) targeted NGS from clinical specimens (no culture required). B) whole genome sequencing from cultured samples. Xpert MTB/RIF (Ultra) is used in this illustrative flow as initial diagnostic test for persons being evaluated for TB.

- 1) If MTB detected / rifampicin resistance not detect, treat with first line regimen, and refer sample for:
  - A) tNGS that may promptly reveal additional resistance and guide appropriate treatment (e.g. for rifampicin-susceptible, isoniazid-resistant TB);
  - B) culture (and phenotypic DST). As soon as culture turns positive, perform WGS that may promptly reveal additional resistance and guide appropriate treatment (e.g. for rifampicin-susceptible, isoniazid-resistant TB), providing extended DST information
- 2) If MTB detected / rifampicin resistance detect, initiate treatment with second line regimen or shorter MDR-TB regimen, as appropriate, and refer sample for:
  - A) tNGS that may promptly reveal additional resistance and guide standardized or individualized MDR/RR-TB regimens;
  - B) culture (and phenotypic DST). As soon as culture turns positive, perform WGS that may promptly reveal additional resistance and guide standardized or individualized MDR/RR-TB regimens, providing extended DST information (including all repurposed and new drugs). WGS approach enables also high-resolution outbreak analysis to guide public health interventions.



(t)NGS: (targeted) Next Generation Sequencing; MTB: *M. tuberculosis*; DST: Drug Susceptibility Testing; MDR: Multidrug resistant; RR: rifampicin-resistant; SNP: Single Nucleotide Polimorphisms; wgSNP: whole genome Single Nucleotide Polimorphisms; cgMLST: core genome Multilocus Sequence Typing  
 \*subjected to batching according to the NGS platform throughput