Real-time sequencing of *Mycobacterium tuberculosis:* are we there yet?

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Abstract

Whole genome sequencing has taken a leading role in epidemiologic studies of tuberculosis, but thus far, the real-time, clinical utility has been low, in part due to the requirement for culture. In Votintseva *et al*. (J Clin Microbiol https://doi.org/10.1128/JCM.02483-16), authors present a new method for extracting *Mycobacterium tuberculosis* DNA directly from smear-positive respiratory samples, generating drug resistance predictions and phylogenetic trees in 44 hours using the Illumina MiSeq. Authors also illustrate the potential for < 24 hour turnaround time from DNA extraction to clinically-relevant results, with Illumina MiniSeq and Oxford Nanopore Technologies' MinION. We comment on the promise and limitations of these approaches.

Whole genome sequencing (WGS) is becoming a mainstay in epidemiologic studies of tuberculosis (TB), with numerous studies demonstrating increased resolution of transmission compared to classical molecular typing methods (reviewed in (1)). While accurately resolving transmission networks is important for public health, it should be noted that countries with the greatest burden of TB often lack resources to conduct such epidemiologic investigations. In such high-incidence, resource-limited settings, diagnosis and treatment remain the greatest priority for TB control. A role for WGS in clinical management of TB has been recently discussed in (2, 3); as comparably inexpensive and rapid tests already available for detection of *Mycobacterium tuberculosis*, the application to drug resistance prediction is where WGS may ultimately have the greatest impact. We have therefore focused this commentary on this facet of care.

Inadequate access to drug susceptibility testing (DST), and long delays in reporting of drug resistance pose serious challenges to TB control. Phenotypic DST is currently the "gold standard" for determining resistance to antibiotics, but can take weeks to months for results as specimens must be sent to centralized laboratories for culture. Given these delays, rapid molecular tests such as Xpert MTB/RIF (Cepheid Inc., Sunnyvale, California, USA) and line probe assays (LPAs) have been endorsed by the World Health Organization, to complement culture-based DST. Although Xpert MTB/RIF and LPAs have enhanced early identification of drug resistance, these tests can miss resistance-conferring mutations that are outside of the locus of interest (e.g., the *rpoB* I491F mutation found to cause rifampin resistance is not detected by Xpert MTB/RIF (4)). They are also unable to differentiate between non-synonymous mutations and synonymous mutations (5), the latter of which are not thought to cause resistance, resulting in false positive predictions of resistance.

In contrast to these targeted, rapid molecular tests, WGS can theoretically identify all mutations in genes associated with resistance at once, and predict the functional effect of these mutations, i.e, whether or not they would likely cause antibiotic resistance. Numerous mutations in the *pncA* gene and inconsistent, unreliable DST (6) suggest that WGS is the best approach for diagnosing resistance to the drug pyrazinamide. This drug is not only a cornerstone of first-line regimens, but also is critical to novel drug combinations (e.g., "BPaZM": bedaquiline, pretomadid, pyrazinamide and moxifloxacin) in late phase trials (7). In addition, WGS offers the potential to detect novel drug mutations; this is of particular interest as new antimicrobials become available and are included in treatment regimens for multi-drug resistant TB (MDR-TB) (7). Using WGS, several resistance-connoting mutations have already been identified for bedaquiline and delamanid (8). With the potential to diagnose and predict drug resistance (as well as delineate transmission networks for public health) all in a single 'test', WGS therefore has the potential to revolutionize TB control programs. To do so, several obstacles must first be overcome in terms of speed (i.e., reducing the turnaround time (TAT) to results), accuracy of predicting phenotypic resistance, and resources requirements.

First, to have the greatest clinical utility, and meet the needs outlined by key stakeholders in the TB community (9), TAT needs to be fast – ideally, with results available the same day as testing. Due to several technical difficulties with performing WGS on clinical samples (reviewed in (10)), thus far, WGS has been predominantly been conducted on *M. tuberculosis* DNA extracted from culture. Using this approach, a head-to-head comparison (11) between WGS and conventional DST found no difference in TAT when real-life delays (as would be experienced in clinical practice) were considered; median TAT from positive Mycobacterial Growth Indicator Tube (MGIT) to WGS-based resistance reports was 31 days (IQR 21-60),

while median time from positive MGIT to DST report was 25 days for conventional DST (interquartile range 14-32). To reduce losses to follow-up, and potentially have an immediate impact on patient care, WGS would ideally be applied directly to clinical samples, as is done with Xpert MTB/RIF.

Thus far, only two studies have been published attempting WGS on such samples (12, 13), with variable success. In a proof-of-concept study published in 2015, Doughty *et al*. extracted *M. tuberculosis* DNA directly from clinical samples and sequenced this using Illumina MiSeq (Illumina, San Diego, USA); however, while TB could be diagnosed, the *M. tuberculosis* DNA obtained was insufficient for drug resistance prediction due to contamination with human DNA (12). A second study published the same year by Brown *et al*. (13) performed targeted enrichment using oligonucleotide baits to capture *M. tuberculosis* DNA prior to sequencing with Illumina MiSeq; this approach yielded ≥20x depth of coverage (the average number of sequencing 'reads' that map to a given position in the reference genome) and >98% genome coverage (the percent of the reference genome with at least one read mapped to it) for 20/24 smear-positive, culture-positive samples (83%). These quality control parameters are in line with early studies that performed WGS on DNA extracted from culture, and were sufficient for speciation and drug resistance prediction. However, this method was expensive and had technical requirements potentially beyond the capacity of most microbiology laboratories.

In this issue of the *Journal of Clinical Microbiology*, Votintseva *et al*. (14) present a new approach for extracting *M. tuberculosis* DNA from direct respiratory specimens, without enrichment. Compared to (13), this method was faster (TAT including MiSeq sequencing was estimated at 50h for Brown *et al*., 44h for Votintseva *et al.*) and less expensive (203 vs

96 Great Britain Pounds per sample for reagents, extraction and sequencing, respectively). All direct samples were correctly identified as *Mycobacterium tuberculosis* complex (95% speciated as *M. tuberculosis*). Overall quality control metrics were lower compared to (13), with depth of coverage >12x and ≥90% genome coverage for only 21/37 (57%) of the smear-positive, culture-positive samples. As direct samples were partitioned for routine clinical use prior to analysis, it is possible this influenced the quantity of *M. tuberculosis* DNA available; however, sample volume was not associated with yield of DNA in univariate and multivariate models.

In addition to the Illumina MiSeq, the authors also tested and timed two other sequencing technologies: the Illumina MiniSeq and the Oxford Nanopore MinION (Oxford Nanopore Technologies, Oxford, UK). For these platforms, DNA was extracted from cultured *Mycobacterium bovis* BCG strain using a previously-reported protocol (15). Pure BCG was then sequenced as well as BCG DNA that was experimentally spiked at specified concentrations (ranging from 5%-15%) into smear-negative, culture-negative sputum. A PCR amplification step, using a new protocol developed by the authors, was performed prior to sequencing with the MinION. The TAT from DNA extraction to complete resistance prediction (and phylogenetic placement) was 16h for MiniSeq, while TAT to these results for a single sample of *M. bovis* BCG DNA spiked at 15% concentration was estimated at 12.5h with the MinION 9.4 flowcell (actual sequencing was performed over 48 hours in total). An advantage of the MinION is that data can be analyzed in real-time; *M. bovis* BCG DNA was detected and correctly identified at 1h, while sequencing was still ongoing. Somewhat disappointingly, authors did not test their novel extraction protocol for direct sequencing of *M. tuberculosis* in combination with either the MiniSeq or MinION. While authors estimate potential TAT for *M. tuberculosis* with the MinION R9.4 flowcell based on the single *M.*

bovis BCG run, to properly assess the performance of the direct sample DNA extraction protocol in combination with this technology, future studies are needed that use real (rather than experimentally-generated) respiratory samples, obtained from patients positive for *M. tuberculosis.* Nonetheless, this study serves as a valuable proof of concept, demonstrating the potential for WGS as a same-day test.

In addition to TAT, accuracy of WGS-based prediction is also a current barrier to implementation; to be financially feasible for most TB control programmes, WGS would optimally serve as a replacement test - eliminating the need for culture altogether. To do so, we need sufficient WGS quality to accurately identify ('call') single nucleotide polymorphisms (SNP; a single change in a base-pair, compared to a reference). The frequency and type of sequencing errors vary across platforms, due to differences in the underlying sequencing chemistry (e.g., Illumina MiSeq has an error rate of 0.8%, PacBio RS 12.9%, MinION 5-30%; Dr. David Dolinger, Foundation for Innovative New Diagnostics, personal communication). Such errors must be accounted for in the analysis, with only high-quality base calls retained. In the case of the MinION, which Votintseva *et al.* applied - for the first time ever reported - to Mycobacterium (14), authors identified a systematic A→G error bias in 1D reads, which must be accounted for in the bioinformatics analysis.

In addition to such platform-specific considerations, overall quality control parameters such as depth of coverage and genome coverage also matter when assessing sequencing results. For such parameters, the minimum thresholds necessary for clinical use still need to be determined. With lower depth of coverage (and/or lower genome coverage), resistance-connoting mutations are more likely to be missed. Using lower thresholds for depth may also reduce our power to detect populations with mixed resistance profiles, or rule out false

positive SNPs due to sequencing or mapping errors. Previous studies performing WGS on DNA from cultured *M. tuberculosis* have required a minimum of 8 to 20x depth of coverage at the specific locus of interest to confidently call a SNP (with a minimum average of 40-50x across the genome currently recommended). Given the technical difficulty of sequencing from direct samples, these thresholds may not be feasible for this approach. In Votintseva *et al.* (14), authors required > 3x depth to allow resistance predictions to be made, a threshold only met by 24/37 (65%) of the direct samples of *M. tuberculosis* sequenced by Illumina MiSeq. Of the 96 possible predictions made for first-line drugs, 92 were concordant with phenotypic DST (96%); the 4 discordant predictions (for rifampin and pyrazinamide) were made on samples from a single patient, which had varying phenotypes for these drugs. When experimentally-spiked *M. bovis* DNA was sequenced using the MinION, the expected H57D *pncA* mutation was confidently called for all but the lowest concentration of BCG DNA (5%). However, while this resistance mutation was correctly classified using a 3x threshold, it is important to note that deeper sequencing was required to confidently exclude SNPs at 174 other loci known to be associated with resistance. Given the consequences of both false positive and false negative resistance calls, before WGS can be implemented into the diagnostic workflow, quality control thresholds used must therefore undergo thorough validation, and ideally, be standardized for reproducible clinical use (16, 17).

In addition to being influenced by WGS data quality, our ability to accurately predict phenotypic resistance is also currently limited by our knowledge of resistance-connoting mutations. The potential for interaction between different mutations, both compensatory and resistance-connoting, as well as strain genetic background (i.e., lineage) (18), further complicates this understanding. While several databases have curated mutations from the literature (e.g., TBDReaMDB (19), TBDR (16)), it is difficult to evaluate causality of such

mutations, and assess for potential interactions absent complete WGS and phenotypic data at the isolate level. Studies using WGS to predict drug resistance ((20-22)) have shown that sensitivity and specificity of this approach varies substantially by drug, with accuracy lowest for second-line anti-TB drugs. As phenotypic DST for second-line drugs is typically only performed if first-line resistance is detected, the numbers of isolates with both genotypic and phenotypic data for these drugs is small. To improve our knowledge of genotypic-phenotypic concordance, large WGS databases with corresponding DST are therefore needed, incorporating both drug-sensitive and drug-resistant isolates (16). In response to this, several multi-national programs have been initiated (e.g.., the CRyPTIC Project at http://modmedmicro.nsms.ox.ac.uk/cryptic/ and ReSeqTB (23) at https://platform.reseqtb.org). These programs are facilitating the collection and curation of WGS data with corresponding phenotypic DST, as well as critical clinical outcome data, from both public and private sectors, which will enhance our understanding of causal resistance-connoting mutations. Until such time, if WGS is used to predict resistance, phenotypic DST should be performed in parallel - a prospect that is likely cost-prohibitive for most TB control programs.

In addition to issues with speed and accuracy, the substantial resources required for WGS are other obstacles to implementation. A large capital investment is necessary to establish the infrastructure for WGS, which may be particularly difficult for developing countries. Sequencing platforms themselves are expensive; as of January 2017, “desktop sequencers” Illumina MiniSeq and MiniSeq cost approximately $50,000 USD and $100,000 USD, respectively (excluding consumables). Platforms with even higher throughput such as the PacBio RS P6-C4 (Pacific Biosciences, Menlo Park, California, USA) or Illumina HiSeq X cost $675,000 USD and $1 million USD, respectively (Dr. David Dolinger, Foundation for

Innovative New Diagnostics, Geneva, personal communication). Aside from the platforms themselves, laboratories must also have allocated space, continuous, uninterrupted power supplies and highly trained personnel to calibrate, operate and support these platforms (24, 25). Oxford Nanopore Technology's MinION, recently used to sequence Ebola strains in Guinea (24), has been proposed as a potential solution to some of these implementation issues. Cost-wise, the Oxford Nanopore MinION platform itself is only $1,000 (price as of February 3rd, 2017, https://store.nanoporetech.com/minion/sets/, including 2 flowcells and a starter kit of reagents). However, a maximum of 12 samples can be sequenced at once using the MinION 9.4 flowcell, and, as they are not reusable, a new flowcell must be used for each additional use (at a cost of ~$10 USD a piece, when purchased in bulk). In terms of laboratory requirements, the MinION is more easily transportable and requires less lab space (24, 25), but still requires human intervention for sample preparation. Thus, regardless of the next-generation sequencing platform used, most of the required resources are currently only be available in a reference laboratory setting, prohibiting the decentralization of WGS for TB diagnostics (26).

In addition to these infrastructure requirements, the bioinformatics analysis of WGS data has been a major bottleneck in the widespread use of this method. Recently, substantial advances in this area have been made, with the development of rapid, user-friendly tools, such as PhyResSE (27), TBProfiler (22), TGS-TB (28) (which uses KvarQ (29)) and Mykrobe Predictor (20), to facilitate resistance prediction. These tools can take raw sequencing files from cultured *M. tuberculosis* DNA all the way through to resistance profiles (though one needs some understanding of bioinformatics concepts for interpretation). Additionally, both Mykrobe and KvarQ can be run offline, though, to our knowledge, Mykrobe is the only rapid resistance tool compatible with the readily-transportable MinION; this is ideal for use in

regions with limited internet access (though periodic updates to include novel resistance-connoting mutations from the literature would be needed). To utilize these tools with the direct sequencing protocol proposed in (14), human DNA must be removed *a priori;* as this involved mapping to a human reference genome, future software iterations will need to automate this step for easier use by frontline technicians without bioinformatics expertise.

In conclusion, WGS has the potential to become the future of TB DST (30) - provided these key considerations in terms of speed, accuracy and resource requirements are addressed. Studies like (14) provide essential methodological advances and proof-of-concept needed to help move WGS to the clinical arena, and ultimately make real-time sequencing of *M. tuberculosis* a reality. To fully evaluate the different DNA extraction protocols for direct sequencing, in combination with different sequencing platforms, future studies are needed employing these methods on the same samples, in a head-to-head comparison. Additional studies are also needed to validate bioinformatics thresholds for clinical use, and assess the performance of direct sequencing on different specimen types (e.g., smear-negative, culture-positive samples, and non-respiratory samples). Given that countries with the highest burden of TB are those with more limited resources, lower-cost, decentralized, simpler platforms are essential for any meaningful scale-up.

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