

# Whole genome sequencing for M/XDR tuberculosis surveillance and for resistance testing

## Abstract

Whole genome sequencing (WGS) can help relate *Mycobacterium tuberculosis* genomes to one another to assess genetic relatedness and infer the likelihood of transmission between cases. The same sequence data is now increasingly being used to predict drug resistance and susceptibility as well. Controlling the spread of tuberculosis and providing patients with the correct treatment are central to the World Health Organization's target to 'End TB' by 2035, to which the global prevalence of drug resistant tuberculosis remains one of the main obstacles to success. WGS has so far been applied largely to drug susceptible strains for the purposes of understanding transmission, leaving a number of analytic considerations before transferring what has been learnt from drug susceptible disease to drug resistant tuberculosis. We discuss these potential problems here, alongside some of the challenges to characterising the *Mycobacterium tuberculosis* 'resistome' – the optimal knowledgebase required for WGS-based assays to successfully direct individualised treatment regimens through the prediction of drug resistance and susceptibility in the future.

## Introduction

In 2014 the World Health Organization announced its 'END TB' strategy. A target was set to reduce global tuberculosis incidence by 90% on 2015 rates by 2035, corresponding to fewer than 10 new cases per 100,000 population per year. 'Intensified research and innovation' was pronounced one of three pillars upon which to build success. There was a call for 'new tools' to be ready by 2025, including a vaccine, better prophylaxis and treatment regimens, and a point of care test. The target was endorsed by all member states at the 2014 World Health Assembly.[1]

As a 'new tool', whole genome sequencing (WGS) is one of the most exciting and disruptive technologies to take to the stage for decades,[2] with advances in nanotechnology now

29 promising to deliver affordable and accessible sequencing platforms that are simple to use, near  
30 the patient.[3] These developments are perhaps more important to the management of  
31 tuberculosis than any other infectious disease due to the diagnostic delays stemming from the  
32 slow growth rate of the *Mycobacterium tuberculosis* complex (Mtb). The introduction of targeted  
33 nucleic acid amplification-based technologies has only partially compensated for this slow growth  
34 rate, notifying clinicians which drugs to avoid, but not which drugs to give the patient.[4]  
35 Consequently, the culture derived phenotype remains the gold standard where practicable and  
36 affordable. Historically, this has left many low income settings relying on just smear microscopy  
37 as the only available diagnostic test, although the roll-out of the Xpert MTB/RIF more recently has  
38 allowed a putative, but helpful, distinction between drug susceptible and multi-drug resistant  
39 (MDR, defined as resistance to at least isoniazid and rifampicin) disease to be drawn.[5]

40 The consequences of empirical or misguided tuberculosis treatment can be disastrous  
41 both at a population, and at an individual level. Under-recognizing and undertreating multi-drug  
42 resistant (MDR) or extensively drug resistant (XDR) tuberculosis at a programmatic level risks  
43 sustaining it in a population through transmission, whereas treating individual tuberculosis  
44 patients with an insufficient number of effective drugs risks selecting for resistant sub-populations,  
45 creating resistant strains *de novo*, or further amplifying the number of drugs ineffective against an  
46 already M/XDR strain. With 480,000 cases of MDR tuberculosis in 2014 already,[6] work towards  
47 a tool for the rapid and accurate diagnosis and control of drug resistance is an absolute priority.  
48 Here we review the prospects of WGS-based technologies providing solutions to both challenges.

## 49 50 Tuberculosis surveillance

51  
52 The 1994 WHO DOTS (Directly Observed Treatment, Short course) strategy called for  
53 the gathering and quarterly reporting of data on cases and outcomes to help optimise control  
54 measures and treatment programmes as epidemiological trends change.[7] Because it is widely  
55 considered that the early detection of outbreaks, and the identification, isolation and treatment of  
56 incident active cases is of value both to the individual concerned and their wider community,

many high-income countries have simultaneously engaged in enhanced surveillance around active cases with the aim of offering chemoprophylaxis to latently infected contacts and treatment to those with active disease.[8] Although the WHO specifically recommends investigating household contacts of patients with infectious MDR tuberculosis in low- and middle-income countries as well, this is largely on an *a priori* basis, as the WHO admits the supporting evidence is poor.[9] With resources often scarce, these recommendations are infrequently followed.

Contact investigations have been aided by classical molecular fingerprinting techniques in high-income countries over the past 25 years. These have included insertion sequence (IS)6110 typing, spoligotyping, and latterly Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeat (MIRU-VNTR) typing, each of which quantify the copy number of relatively stable repetitive segments of DNA in the *Mtbc* genome.[10] By dividing culture confirmed cases into discrete groups, typing techniques have allowed cluster investigations to search for epidemiological links between cases with similar fingerprints, even though similar fingerprints by themselves are insufficient grounds to expect a link. The well documented advantages of WGS data are that they provide greater resolution than traditional techniques,[11-13] allowing contact investigations to be directed with greater specificity. Despite the obvious potential for cost saving, there has been no study to date to formally assesses the impact of WGS on tuberculosis control in general, or on M/XDR disease in particular. Moreover, options for public health interventions in M/XDR outbreaks are potentially fewer than for drug susceptible disease, with the precise role for chemoprophylaxis remaining controversial, not least because M/XDR-tuberculosis is a heterogeneous entity that can be resistant to many different drugs.[14]

How WGS might help in low and medium-income settings has also still to be defined and assessed. Intuitively, constrained resources would most sensibly be targeted at transmission of M/XDR disease with a view to active case finding. Because drug resistance can be predicted from the genome sequence prior to phenotypic results becoming available, transmission of M/XDR strains could be detected early,[15] potentially facilitating more rapid public health responses, including active case finding. However, although it is expected that WGS will help focus cluster investigations on the most relevant cases, it remains an assumption that this will

85 lead to less transmission of disease and improved health outcomes.

86 To maximise the potential benefit of WGS for surveillance of M/XDR-tuberculosis, what  
87 has been learned from drug susceptible disease must be accurately applied to and adjusted for  
88 the peculiarities of M/XDR disease. This includes the interpretation of genomic distances, and the  
89 understanding of mutation rates, lineage effects and host-dependent factors such as HIV.

90 Approaches to WGS data analysis will no doubt be further honed for maximal  
91 epidemiological gain, but to date there has been a remarkable convergence of results emanating  
92 from different research groups, using a diversity of analytic techniques. In the vast majority of  
93 cases direct or recent transmission has been associated with only a handful of nucleotide  
94 differences separating samples.[11,12,16-18] Pragmatic single nucleotide polymorphism (SNP)  
95 thresholds have therefore been proposed to help guide cluster investigations,[16] helping  
96 accommodate some variability that has been observed in the molecular clock over short time  
97 periods.[17] It has also been argued in multiple independent reports that phylogenetic signals  
98 could be used to infer the direction of transmission between patients,[16,19-21] providing further  
99 advantage over one-dimensional fingerprinting results by helping target investigations at the  
100 contacts of the most infectious patients. Each of these findings have contributed to the decision to  
101 implement WGS into routine practice in England, and towards similar plans in other high-income  
102 countries.[22] The contribution towards tuberculosis control will be assessed as a matter of  
103 process in these countries where the incidence and rates of M/XDR-tuberculosis tend to be low,  
104 but from which lessons for the control of M/XDR disease may be learnt.

105 The idea of a consistent molecular clock is a convenient generalisation across genomic  
106 loci and across lineages within the Mtb, but there are reasons M/XDR disease might be  
107 considered separately due to factors potentially accelerating the clock. Genomic loci under  
108 positive selection pressure, including those conferring drug resistance or compensating for the  
109 fitness cost of resistance, have a higher than background mutation rate.[4] The phenomenon of  
110 'hitch-hiking' SNPs has also been described, where SNPs involved in drug resistance co-emerge  
111 with others that play no ostensive role in resistance.[23] Loci like these with higher mutation rates  
112 may result in a greater than expected number of SNPs being observed between patients where

113 resistance is acquired. The problem may be compounded where patients suffering prolonged  
114 infection develop divergent microevolution at different anatomical sites, possibly influenced by  
115 exposure to treatment, and potentially resulting in greater within host pathogen diversity than has  
116 come to be expected (Figure 1).[16,24] Inferences about transmission must thus also take patient  
117 sampling into account. Each of these factors has the potential to alter the expected number of  
118 SNPs between patients potentially linked by recent M/XDR transmission.

119         One approach to mitigating the effect of a faster molecular clock at loci that are under  
120 positive selection pressure has been to leave out sites associated with drug resistance when  
121 assessing transmission.[11] This could allow interpretative parameters derived from drug  
122 susceptible disease to be applied to M/XDR disease. However, which sites to leave out is not  
123 obvious, as many mutations are only putatively associated with resistance,[4] whilst others sites  
124 under selection may be compensatory mutations, 'hitch-hiking' SNPs, or those under selection  
125 from immune pressure. Studies focussing on the transmission of M/XDR strains are therefore  
126 urgently needed to explore whether the phenomena described here are widespread, and whether  
127 the experience from patients with drug susceptible tuberculosis can be applied.

128         Lineage and host effects may prove to be further confounders. Differential rates of  
129 emergence of drug resistance mutations have been documented between the East Asian  
130 (Beijing) and Euro-American lineages.[25] The Beijing lineage has been linked to the emergence  
131 of MDR disease in Eurasia over the past 30 years, as well as to highly resistant clones in South  
132 Africa where its success has been linked to the emergence of the HIV pandemic.[26-28] How HIV  
133 has contributed to the success of MDR tuberculosis remains unclear. One hypothesis is that HIV  
134 treatment influences the pathogen mutation rate, but this is both unproven and could not account  
135 for events preceding the mass-treatment era or HIV pandemic .[29] Indeed, it has been argued  
136 that Latin-American-Mediterranean (LAM4) lineage strains from the Tugela Ferry outbreak in  
137 South Africa acquired drug resistance well before the HIV pandemic.[32] An alternative  
138 hypothesis argues that drug resistant strains manage to successfully infect patients living with  
139 HIV despite potentially bearing some fitness costs, thereby reducing purifying selection and  
140 accelerating microevolution and the acquisition of drug resistance.[29-31] However, a recent

study of a prolonged outbreak of MDR tuberculosis in South America did not demonstrate an HIV-effect on the pathogen mutation rate.[33]

For the robust comparison of sequenced M/XDR strains, including across jurisdictions, standardised data analysis pipelines will be necessary. Consideration will have to be given to the acquisition of drug resistance, lineage, and maybe even to host factors if the time to the most recent common ancestor is to be accurately predicted for potentially clustered M/XDR strains. One approach to achieving standardisation in general, rather than for M/XDR-tuberculosis in particular, has been to construct an easily comparable genetic bar code based on gene alleles, at the expense of some resolution.[21] Achieving comparability of data without the loss of resolution however remains a preferable option and a research objective. Whatever the eventual solution, more work will be needed to assess the universality of previously defined criteria for identifying recent transmission, be that for outbreaks with evolving drug resistance or for outbreaks of circulating clones with established M/XDR patterns.[28,34] The future contribution of WGS towards the control of M/XDR-tuberculosis, and thereby to the elimination of tuberculosis altogether, therefore remains uncertain, even though there are reasons to be confident.

#### Predicting drug resistance and susceptibility

It is estimated that only 25% of the 480,000 worldwide cases of MDR-TB were detected and reported in 2014, largely because the costs of laboratory based phenotyping are beyond many low income settings.[6] That proportion has been increasing since the WHO endorsed the Xpert MTB/RIF in 2011,[35] but as with all other molecular assays based on targeted amplification,[5] the Xpert MTB/RIF predicts resistance, and not susceptibility, informing a clinician which drugs to avoid, rather than which drugs to give. With the WHO's target to end TB by 2035, research activity into how WGS could form the basis of a new diagnostic test that predicts both resistance and susceptibility, whilst differentiating between newly evolved and transmitted M/XDR-tuberculosis, is now increasing (Figure 2).[36]

Much is already known about the molecular determinants of resistance to the first-line

169 drugs, including isoniazid and rifampicin, whereas the determinants of resistance to pyrazinamide  
170 and 3<sup>rd</sup> and 4<sup>th</sup> line drugs are less well described.[4,37] Even once the range of determinants of  
171 drug resistance is more completely understood, the contribution of WGS to achieving the WHO  
172 target will depend on whether personalized treatment regimens can deliver improved outcomes  
173 for M/XDR patients, at a programmatic level, when compared to 'one-size-fits-all' treatment  
174 protocols such as the recently WHO endorsed 'Bangladesh regimen'. [38] With current  
175 technologies, implementation of personalized treatment regimens will remain difficult in many low-  
176 income settings where phenotypic drug susceptibility testing remains unavailable due a lack of  
177 infrastructure and trained staff.[6] A paucity of doctors further compounds the problem in these  
178 settings as bespoke regimens, by their very nature, require design. However, portable WGS  
179 platforms provide an opportunity to translate expanding knowledge of the molecular determinants  
180 of resistance into field technology that could circumvent the need for complex infrastructure.[3] It  
181 is however possible to foresee how data analysis platforms could be combined with suitable  
182 algorithms to define personalized regimens, reducing dependence on medically qualified staff.

183 WGS-based individualisation of therapy for the treatment of M/XDR disease will require  
184 an extensive knowledgebase characterising the effect of genomic mutations on all relevant drugs.  
185 However, a clear understanding of what the molecular-level data is predicting is essential. WGS  
186 studies to date have attempted to link particular mutations to minimum inhibitory concentrations  
187 (MICs),[39] or to a probability of one of two outcomes – phenotypic resistance or  
188 susceptibility.[37] Analytic approaches to large data sets have ranged from the heuristic to the  
189 mathematically principled,[4,37,40,41] whilst laboratory approaches have ranged from passage  
190 and selection of spontaneous mutations to targeted mutagenesis.[42,43] But whilst some  
191 mutations have been identified as predictive of the *in vitro* phenotype,[4] this is itself only a proxy  
192 for overall clinical outcome. The aspiration would be for molecular data to predict outcome  
193 directly, as challenging as this may prove to be (Figure 3). The clinical implications of some  
194 mutations known to lead to high-level isoniazid (katG<sup>315</sup>), rifampicin (rpoB<sup>S450L</sup>) and  
195 fluoroquinolone resistance (gyrA<sup>94</sup>) and treatment failure have recently been outlined in a  
196 consensus statement.[44] The clinical significance of other mutations conferring lower-level

197 resistance, such as inhA promotor region mutations, and at rpoB<sup>H445L</sup>, is less clear.[44,45]  
198 However, whilst phenotypes themselves are established predictors of outcome in some  
199 cases,[46] in many cases such a correlation remains to be established despite this being  
200 ostensibly a lesser challenge than linking individual mutations to outcome.[47] Not only has  
201 phenotypic data been collected over a longer time period, it is often presented in binary or ternary  
202 terms (susceptible / low level resistance / high level resistance), rather than as quantitative  
203 data.[48] Linking the individual or combined role of a plethora of mutations to clinical outcome will  
204 be more complicated.

205         A significant amount of resistance to anti-tuberculosis drugs can be reduced to the effects  
206 of single mutations in the form of SNPs and small insertions or deletions.[4] This holds true for  
207 other members of the *M. tuberculosis* complex such as *Mycobacterium africanum*, although  
208 similar mutations have been observed to arise with different frequencies.[49] More complex  
209 mechanisms of resistance have also been described, with compensatory mutations mitigating the  
210 fitness costs of resistance conferring mutations,[50] and that the effects of multiple mutations  
211 cumulatively leading to stepwise increases in MIC.[51] Mutations can also be antagonistic  
212 (epistatic), where the pre-existence of one mutation cancels out the effect of another, as with  
213 gyrA<sup>90</sup> mutations in the Uganda lineage that bearing a background SNP at gyrA<sup>80</sup>. [52] Other  
214 mechanisms include efflux pumps whose activity can be predicted by detection of genomic  
215 mutation, as for bedaquiline,[53] but whose activity can also be determined by epigenetic factors,  
216 such as drug exposure,[23] not detectable by WGS.

217         A further complication – but also opportunity – is posed by the detection of minority  
218 bacterial populations, represented as minority variants. This is where the aligned sequencing  
219 reads don't all agree on a particular nucleotide, potentially indicating the presence of a sub-  
220 clone.[24] With insufficient sequencing depth such minority species can be missed, to potential  
221 detriment if they go on to be selected for under drug pressure. To match the proportion method  
222 for detecting drug resistance in bacterial culture, which is designed to detect resistant  
223 subpopulations accounting for 1% or more of the total population,[54] a minimum acceptable  
224 sequencing read depth will have to be achieved. One challenge for the future will be to perform a



Bayesian risk assessment to offset the chance of missing minority variants at any given read depth against the safety-net provided by combination therapy.

Software solutions to detect drug resistance mutations are already available, but each is only as accurate as its integrated knowledgebase.[55-57] Collectively these provide an assurance that the screening of a sample for characteristic mutations will not be the largest challenge ahead when diagnosing M/XDR-tuberculosis. Indeed, they provide an opportunity to phase in WGS-based diagnostics, as is happening in England,[15,22] with the aim to phase out routine phenotyping, reserving it only for strains bearing novel or insufficiently well characterized mutations (only about 10% of new strains have been found to have novel mutation across relevant genes).[4] As the knowledgebase grows, the number of phenotypic assays required is expected to wane, although some background DST will need to continue if emerging resistance is to be detected.

## Conclusion

Despite an incomplete understanding of the molecular determinants of M/XDR-tuberculosis, such is the demand for better molecular diagnostics that WGS data is already being sporadically used to guide patient management, even where phenotypic susceptibility data is available.[58] However, until the molecular determinants of resistance are more completely understood, phenotyping will have to continue. The creation of public databases through which genotypic, phenotypic and outcome-data can be shared, will help to standardise genome based diagnostics and accelerate the transition from phenotypic to genotypic resistance prediction. The number of papers published on the use of WGS for tuberculosis outbreak control over the past five years is testament to the enthusiasm with which this technology is being received. It appears very likely that demand for WGS will continue to increase even as essential research and development remains in progress over the coming years (Figure 3). However, the community will have to eventually converge on standardised approaches to data analysis to ensure maximum comparability. The likely impact on preventing transmission of M/XDR-tuberculosis, and on

improving diagnostic speed and precision, needs to be widely assessed. However, the experience of the Xpert MTB/RIF assay roll-out underlines the need to strengthen health systems in parallel to the implementation of any new test if the 2035 target is to be met.[59]

Transparency declaration:

None of the authors have any conflict of interest. TMW is a National Institute of Health Research (NIHR) Academic Clinical Lecturer and DWC and TEAP are NIHR Senior Investigators supported by the NIHR Oxford Biomedical Research Centre (BRC) programme. SN is a consultant to FIND. All authors contributed to the design and writing of this review.

Title and legend to figure 1:

'Factors influencing molecular clock and genetic diversity in M/XDR tuberculosis'

Panel A and B: Population based effects. Panel A shows a phylogenetic tree of the Mtb lineages with the 'Beijing' lineage, purported to have a higher mutation rate, coloured red. Panel B is a cartoon showing the emergence of the same example mutation independently on different branches of the tree ('homoplasy') due to positive selection.

Panel C and D: Evolution within the individual. Panel C shows a cartoon of the circular Mtb genome / chromosome in which a single drug resistance mutation (in red) arises and is accompanied by compensatory or hitchhiking mutations (in blue), accelerating the rate of microevolution. Panel D shows a cartoon of the evolution of Mtb strains within two hosts, (i) and (ii), infected by the same source at the same time point, 0. As patient (i) is not diagnosed until time point  $t$ , there has been opportunity for greater within host evolution, and for the spontaneous emergence of M/XDR conferring mutations. Conclusions about the relatedness to case (ii) will be influenced by which strain is sampled from (i), and when.

Figure 2 title:

'Comparison of currently available diagnostic modalities to nanotechnology based WGS'

Figure 3 title:

'Advantages and remaining challenges of WGS for surveillance and susceptibility testing'

## References

- [1] The End TB Strategy. WhoInt n.d. [http://who.int/tb/post2015\\_TBstrategy.pdf?ua=1](http://who.int/tb/post2015_TBstrategy.pdf?ua=1) (accessed January 3, 2015).
- [2] Didelot X, Bowden R, Wilson DJ, Peto TEA, Crook DW. Transforming clinical microbiology with bacterial genome sequencing. *Nat Rev Genet* 2012;13:601–12. doi:10.1038/nrg3226.
- [3] Quick J, Loman NJ, Duraffour S, Simpson JT, Severi E, Cowley L, et al. Real-time, portable genome sequencing for Ebola surveillance. *Nature* 2016;530:228–32. doi:10.1038/nature16996.
- [4] Walker TM, Kohl TA, Omar SV, Hedge J, Del Ojo Elias C, Bradley P, et al. Whole-genome sequencing for prediction of *Mycobacterium tuberculosis* drug susceptibility and resistance: a retrospective cohort study. *Lancet Infect Dis* 2015. doi:10.1016/S1473-3099(15)00062-6.
- [5] Scott LE, McCarthy K, Gous N, Nduna M, Van Rie A, Sanne I, et al. Comparison of Xpert MTB/RIF with other nucleic acid technologies for diagnosing pulmonary tuberculosis in a high HIV prevalence setting: a prospective study. *Plos Med* 2011;8:e1001061. doi:10.1371/journal.pmed.1001061.
- [6] World Health Organization. Global Tuberculosis Report 2015, 20th edition n.d. [http://apps.who.int/iris/bitstream/10665/191102/1/9789241565059\\_eng.pdf?ua=1](http://apps.who.int/iris/bitstream/10665/191102/1/9789241565059_eng.pdf?ua=1) (accessed July 3, 2016).
- [7] Programme WGT. An Expanded DOTS Framework for Effective Tuberculosis Control. 2002.
- [8] National Institute for Clinical Excellence. Tuberculosis 2016:1–178.
- [9] Hopewell PC, Fair E, Miller C. Recommendations for Investigating Contacts of Persons with Infectious Tuberculosis in Low- and Middle-Income Countries. 2012.
- [10] Niemann S, Supply P. Diversity and evolution of *Mycobacterium tuberculosis*: moving to whole-genome-based approaches. *Cold Spring Harbor Perspectives in Medicine* 2014;4:a021188–8. doi:10.1101/cshperspect.a021188.
- [11] Roetzer A, Diel R, Kohl TA, Rückert C, Nübel U, Blom J, et al. Whole Genome Sequencing versus Traditional Genotyping for Investigation of a *Mycobacterium tuberculosis* Outbreak: A Longitudinal Molecular Epidemiological Study. *Plos Med* 2013;10:e1001387. doi:10.1371/journal.pmed.1001387.
- [12] Walker TM, Lalor MK, Broda A, Saldana Ortega L, Morgan M, Parker L, et al. Assessment of *Mycobacterium tuberculosis* transmission in Oxfordshire, UK, 2007–12, with whole pathogen genome sequences: an observational study. *The Lancet Respiratory Medicine* 2014;2:285–92. doi:10.1016/S2213-2600(14)70027-X.
- [13] Guerra-Assunção JA, Crampin AC, Houben R, Mzembe T, Mallard K, Coll F, et al. Large-scale whole genome sequencing of *M. tuberculosis* provides insights into transmission in a high prevalence area. *eLife Sciences* 2015;4:e05166. doi:10.7554/eLife.05166.
- [14] Chiappini E, Sollai S, Bonsignori F, Galli L, de Martino M. Controversies in preventive therapy for children contacts of multidrug-resistant tuberculosis. *J Chemother* 2014;26:1–12. doi:10.1179/1973947813Y.0000000105.
- [15] Pankhurst LJ, Del Ojo Elias C, Votintseva AA, Walker TM, Cole K, Davies J, et al. Rapid, comprehensive, and affordable mycobacterial diagnosis with whole-genome sequencing: a prospective study. *The Lancet Respiratory Medicine* 2016;4:49–58. doi:10.1016/S2213-2600(15)00466-X.
- [16] Walker TM, Ip CL, Harrell RH, Evans JT, Kapatai G, Dedicoat MJ, et al. Whole-genome sequencing to delineate *Mycobacterium tuberculosis* outbreaks: a retrospective observational study. *Lancet Infect Dis* 2013;13:137–46. doi:10.1016/S1473-3099(12)70277-3.
- [17] Bryant JM, Schurch AC, van Deutekom H. Inferring patient to patient transmission of *Mycobacterium tuberculosis* from whole genome sequencing data. *BMC Infectious ...* 2013.
- [18] Guerra-Assunção JA, Houben RMGJ, Crampin AC, Mzembe T, Mallard K, Coll F, et

al. Recurrence due to Relapse or Reinfection With *Mycobacterium tuberculosis*: A Whole-Genome Sequencing Approach in a Large, Population-Based Cohort With a High HIV Infection Prevalence and Active Follow-up. *JidOxfordjournalsorg* n.d.

[19] Kato-Maeda M, Ho C, Passarelli B, Banaei N, Grinsdale J, Flores L, et al. Use of Whole Genome Sequencing to Determine the Microevolution of *Mycobacterium tuberculosis* during an Outbreak. *PLoS ONE* 2013;8:e58235. doi:10.1371/journal.pone.0058235.s002.

[20] Stucki D, Ballif M, Bodmer T, Coscolla M, Maurer A-M, Droz S, et al. Tracking a Tuberculosis Outbreak Over 21 Years: Strain-Specific Single-Nucleotide Polymorphism Typing Combined With Targeted Whole-Genome Sequencing n.d.

[21] Kohl TA, Diel R, Harmsen D, Rothgänger J, Walter KM, Merker M, et al. Whole-Genome-Based *Mycobacterium tuberculosis* Surveillance: a Standardized, Portable, and Expandable Approach n.d.

[22] Luheshi L, Raza S, Moorthie S, Hall A, Blackburn L, Rands C, et al. ***Pathogen Genomics Into Practice PHG Foundation (2015) ISBN 978-1-907198-18-2***

. 2015.

[23] Eldholm V, Norheim G, Lippe von der B, Kinander W, Dahle UR, Caugant DA, et al. Evolution of extensively drug-resistant *Mycobacterium tuberculosis* from a susceptible ancestor in a single patient. *Genome Biology* 2014;15:490–0. doi:10.1186/s13059-014-0490-3.

[24] Liu Q, Via LE, Luo T, Liang L, Liu X, Wu S, et al. Within patient microevolution of *Mycobacterium tuberculosis* correlates with heterogeneous responses to treatment. *Sci Rep* 2015;5:17507. doi:10.1038/srep17507.

[25] Ford CB, Shah RR, Maeda MK, Gagneux S, Murray MB, Cohen T, et al. *Mycobacterium tuberculosis* mutation rate estimates from different lineages predict substantial differences in the emergence of drug-resistant tuberculosis. *Nat Genet* 2013;–. doi:doi:10.1038/ng.2656.

[26] Casali N, Nikolayevskyy V, Balabanova Y, Harris SR, Ignatyeva O, Kontsevaya I, et al. Evolution and transmission of drug-resistant tuberculosis in a Russian population. *Nat Genet* 2014;46:279–86. doi:10.1038/ng.2878.

[27] Merker M, Blin C, Mona S, Duforet-Frebourg N, Lecher S, Willery E, et al. Evolutionary history and global spread of the *Mycobacterium tuberculosis* Beijing lineage. *Nat Genet* 2015;47:242–9. doi:10.1038/ng.3195.

[28] Iöerger TR, Feng Y, Chen X, Dobos KM, Victor TC, Streicher EM, et al. The non-clonality of drug resistance in Beijing-genotype isolates of *Mycobacterium tuberculosis* from the Western Cape of South Africa. *BMC Genomics* 2010;11:670. doi:10.1186/1471-2164-11-670.

[29] McGrath M, Gey van Pittius NC, van Helden PD, Warren RM, Warner DF. Mutation rate and the emergence of drug resistance in *Mycobacterium tuberculosis*. *J Antimicrob Chemother* 2014;69:292–302. doi:10.1093/jac/dkt364.

[30] Sergeev R, Colijn C, Murray M, Cohen T. Modeling the dynamic relationship between HIV and the risk of drug-resistant tuberculosis. *Science Translational Medicine* 2012;4:135ra67–7. doi:10.1126/scitranslmed.3003815.

[31] Strauss OJ, Warren RM, Jordaan A, Streicher EM, Hanekom M, Falmer AA, et al. Spread of a low-fitness drug-resistant *Mycobacterium tuberculosis* strain in a setting of high human immunodeficiency virus prevalence. *Journal of Clinical Microbiology* 2008;46:1514–6. doi:10.1128/JCM.01938-07.

[32] Cohen KA, Abeel T, Manson McGuire A, Desjardins CA, Munsamy V, Shea TP, et al. Evolution of Extensively Drug-Resistant Tuberculosis over Four Decades: Whole Genome Sequencing and Dating Analysis of *Mycobacterium tuberculosis* Isolates from KwaZulu-Natal. *Plos Med* 2015;12:e1001880–22. doi:10.1371/journal.pmed.1001880.

[33] Eldholm V, Rieux A, Monteserin J, Montana Lopez J, Palmero D, Lopez B, et al. Impact of HIV co-infection on the evolution and transmission of multidrug-resistant tuberculosis 2016:1–19. doi:10.7554/eLife.16644.001.

402 [34] Lanzas F, Karakousis PC, Sacchetti JC, Iorger TR. Multidrug-resistant  
403 tuberculosis in panama is driven by clonal expansion of a multidrug-resistant  
404 *Mycobacterium tuberculosis* strain related to the KZN extensively drug-resistant M.  
405 *tuberculosis* strain from South Africa. *Journal of Clinical Microbiology*  
406 2013;51:3277–85. doi:10.1128/JCM.01122-13.

407 [35] Weyer K. Automated Real-time Nucleic Acid Amplification Technology for Rapid  
408 and Simultaneous Detection of Tuberculosis and Rifampicin Resistance: Xpert  
409 MTB/RIF System 2012:1–36.

410 [36] Global team aim faster, more effective TB diagnosis. OxAcUk n.d.  
411 [http://www.ox.ac.uk/news/2016-03-24-global-team-aim-faster-more-effective-tb-](http://www.ox.ac.uk/news/2016-03-24-global-team-aim-faster-more-effective-tb-diagnosis)  
412 [diagnosis](http://www.ox.ac.uk/news/2016-03-24-global-team-aim-faster-more-effective-tb-diagnosis) (accessed July 3, 2016).

413 [37] Miotto P, Cabibbe AM, Feuerriegel S, Casali N, Drobniewski F, Rodionova Y, et al.  
414 *Mycobacterium tuberculosis* Pyrazinamide Resistance Determinants: a Multicenter  
415 Study. *MbioAsmorg* n.d.

416 [38] World Health Organization, World. THE SHORTER MDR-TB REGIMEN 2016:1–2.  
417 [http://www.who.int/tb/Short\\_MDR\\_regimen\\_factsheet.pdf](http://www.who.int/tb/Short_MDR_regimen_factsheet.pdf) (accessed July 3, 2016).

418 [39] Jamieson FB, Guthrie JL, Neemuchwala A, Lastovetska O, Melano RG, Mehaffy C.  
419 Profiling of rpoB Mutations and MICs for Rifampin and Rifabutin in *Mycobacterium*  
420 *tuberculosis*. *JcmAsmorg* n.d.

421 [40] Farhat MR, Sultana R, Iartchouk O, Bozeman S, Galagan J, Sisk P, et al. Genetic  
422 Determinants of Drug Resistance in *Mycobacterium tuberculosis* and Their  
423 Diagnostic Value. *American Journal of Respiratory and Critical Care Medicine* 2016.  
424 doi:10.1164/rccm.201510-2091OC.

425 [41] Earle SG, Wu C-H, Charlesworth J, Stoesser N, Gordon NC, Walker TM, et al.  
426 Identifying lineage effects when controlling for population structure improves power  
427 in bacterial association studies. *Nature Microbiology* 2016;1:16041.  
428 doi:10.1038/nmicrobiol.2016.41.

429 [42] McGrath M, Gey van Pittius NC, Sirgel FA, Van Helden PD, Warren RM.  
430 Moxifloxacin retains antimycobacterial activity in the presence of gyrA mutations.  
431 *Antimicrob Agents Chemother* 2014;58:2912–5. doi:10.1128/AAC.02583-13.

432 [43] Nebenzahl-Guimaraes H, Jacobson KR, Farhat MR, Murray MB. Systematic review  
433 of allelic exchange experiments aimed at identifying mutations that confer drug  
434 resistance in *Mycobacterium tuberculosis* n.d.

435 [44] Dominguez J, Boettger EC, Cirillo D, Cobelens F, Eisenach KD, Gagneux S, et al.  
436 Clinical implications of molecular drug resistance testing for *Mycobacterium*  
437 *tuberculosis*: a TBNET/RESIST-TB consensus statement. *Int J Tuberc Lung Dis*  
438 2016;20:24–42. doi:10.5588/ijtld.15.0221.

439 [45] Huyen MNT, Cobelens FGJ, Buu TN, Lan NTN, Dung NH, Kremer K, et al.  
440 Epidemiology of Isoniazid Resistance Mutations and Their Effect on Tuberculosis  
441 Treatment Outcomes. *AacAsmorg* n.d.

442 [46] STEWART SM, CROFTON JW. THE CLINICAL SIGNIFICANCE OF LOW  
443 DEGREES OF DRUG RESISTANCE IN PULMONARY TUBERCULOSIS. *Am Rev*  
444 *Respir Dis* 1964;89:811–29. doi:10.1164/arrd.1964.89.6.811.

445 [47] Zheng X, Zheng R, Hu Y, Werngren J, Davies Forsman L, Mansjö M, et al.  
446 Determination of Minimum Inhibitory Concentration (MIC) Breakpoints for second-  
447 line drugs associated with clinical outcomes in multidrug-resistant tuberculosis  
448 treatment in China. *Antimicrob Agents Chemother* 2016:AAC.03008–15.  
449 doi:10.1128/AAC.03008-15.

450 [48] Böttger EC. The ins and outs of *Mycobacterium tuberculosis* drug susceptibility  
451 testing. *Clin Microbiol Infect* 2011;17:1128–34.

452 [49] Otchere ID, Asante-Poku A, Osei-Wusu S, Baddoo A, Sarpong E, Ganiyu AH, et al.  
453 Detection and characterization of drug-resistant conferring genes in *Mycobacterium*  
454 *tuberculosis* complex strains: A prospective study in two distant regions of Ghana.  
455 *Tuberculosis* 2016;99:147–54. doi:10.1016/j.tube.2016.05.014.

456 [50] Comas I, Borrell S, Roetzer A, Rose G, Malla B, Kato-Maeda M, et al. Whole-  
457 genome sequencing of rifampicin-resistant *Mycobacterium tuberculosis* strains

458 identifies compensatory mutations in RNA polymerase genes. *Nat Genet*  
 459 2011;44:106–10. doi:10.1038/ng.1038.  
 460 [51] Safi H, Lingaraju S, Amin A, Kim S, Jones M, Holmes M, et al. Evolution of high-  
 461 level ethambutol-resistant tuberculosis through interacting mutations in  
 462 decaprenylphosphoryl. *Nat Genet* 2013;45:1190–7. doi:10.1038/ng.2743.  
 463 [52] Pantel A, Petrella S, Veziris N, Matrat S, Bouige A, Ferrand H, et al. Description of  
 464 compensatory gyrA mutations restoring fluoroquinolone susceptibility in  
 465 *Mycobacterium tuberculosis*. *J Antimicrob Chemother* 2016:dkw169.  
 466 doi:10.1093/jac/dkw169.  
 467 [53] Almeida D, Ioerger T, Tyagi S, Li S-Y, Mdluli K, Andries K, et al. Mutations in pepQ  
 468 Confer Low-level Resistance to Bedaquiline and Clofazimine in *Mycobacterium*  
 469 *tuberculosis*. *Antimicrob Agents Chemother* 2016:AAC.00753–16.  
 470 doi:10.1128/AAC.00753-16.  
 471 [54] Drobniewski F, Rusch-Gerdes S, Hoffner S, Subcommittee on Antimicrobial  
 472 Susceptibility Testing of *Mycobacterium tuberculosis* of the European Committee for  
 473 Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical  
 474 Microbiology and Infectious Diseases (ESCMID). Antimicrobial susceptibility testing  
 475 of *Mycobacterium tuberculosis* (EUCAST document E.DEF 8.1)--report of the  
 476 Subcommittee on Antimicrobial Susceptibility Testing of *Mycobacterium*  
 477 *tuberculosis* of the European Committee for Antimicrobial Susceptibility Testing  
 478 (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases  
 479 (ESCMID). *Clin Microbiol Infect* 2007;13:1144–56. doi:10.1111/j.1469-  
 480 0691.2007.01813.x.  
 481 [55] Bradley P, Gordon NC, Walker TM, Dunn L, Heys S, Huang B, et al. Rapid antibiotic  
 482 resistance predictions from genome sequence data for *S. aureus* and *M.*  
 483 *tuberculosis*. *Cold Spring Harbor Labs Journals*; 2015. doi:10.1101/018564.  
 484 [56] Steiner A, Stucki D, Coscolla M, Borrell S, Gagneux S. KvarQ: targeted and direct  
 485 variant calling from fastq reads of bacterial genomes. *BMC Genomics* 2014;15:881.  
 486 doi:10.1186/1471-2164-15-881.  
 487 [57] Feuerriegel S, Schleusener V, Beckert P, Kohl TA, Miotto P, Cirillo DM, et al.  
 488 PhyResSE: a Web Tool Delineating *Mycobacterium tuberculosis* Antibiotic  
 489 Resistance and Lineage from Whole-Genome Sequencing Data. *Journal of Clinical*  
 490 *Microbiology* 2015;53:1908–14. doi:10.1128/JCM.00025-15.  
 491 [58] Witney AA, Gould KA, Arnold A, Coleman D, Delgado R, Dhillon J, et al. Clinical  
 492 application of whole-genome sequencing to inform treatment for multidrug-resistant  
 493 tuberculosis cases. *Journal of Clinical Microbiology* 2015;53:1473–83.  
 494 doi:10.1128/JCM.02993-14.  
 495 [59] Churchyard GJ, Stevens WS, Mametja LD, McCarthy KM, Chihota V, Nicol MP, et  
 496 al. Xpert MTB/RIF versus sputum microscopy as the initial diagnostic test for  
 497 tuberculosis: a cluster-randomised trial embedded in South African roll-out of Xpert  
 498 MTB/RIF. *The Lancet Global Health* 2015;3:e450–7. doi:10.1016/S2214-  
 499 109X(15)00100-X.  
 500