

Seeking the source of bacterial drug resistance

A targeted deep sequencing assay identifies multidrug-resistant tuberculosis strains responsible for silent outbreaks



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Tuberculosis (TB) is often thought of as a disease of the past. Yet, this potentially lethal medical affliction, once known as “consumption,” remains a leading cause of death globally, especially in low- and lower-middle-income countries.¹ The COVID-19 pandemic has stalled and reversed progress made on access to TB diagnosis and treatment over the past years.² According to the World Health Organization (WHO), an estimated 10.6 million people fell ill with TB in 2021 and 1.6 million perished from the disease.² Its current predominance stems in part from the bacterium’s ability to evolve, even when faced with a six-month treatment regimen of four specific antimicrobial drugs. Such treatment has been used for decades and, over time, many TB strains have developed resistance to once effective first-line drugs such as isoniazid and rifampicin. In 2021, an estimated 450,000 new TB cases were resistant to rifampicin or to both isoniazid and rifampicin,² representing multidrug resistance (MDR) and making the disease that much harder to treat and more likely to spread. Early detection of TB outbreaks and effective identification of new MDR-TB strains have become essential in combating the disease.

Philip Supply, PhD, a senior scientist and research director of the French National Center for Scientific Research (CNRS) at the Center for Infection and Immunity at the Institut Pasteur de Lille, has spent most of his career studying the bacterium that causes TB, *Mycobacterium tuberculosis*. As a scientific consultant for GenoScreen, a French biotech company that provides state-of-the-art sequencing and genomic analysis services, Dr. Supply assisted in the development of a novel targeted deep sequencing assay, Deeplex Myc-TB.³ Sensitive and accurate, this assay can identify drug-resistant TB strains directly from clinical sputum samples, without the need for time-consuming bacterial cultures.³

We spoke with Dr. Supply about the growing problem of MDR-TB, the value that Illumina next-generation sequencing (NGS) systems bring to their detection, development of the Deeplex Myc-TB assay, and how it could be used to detect MDR-TB strains and limit silent outbreaks.

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Q: What sparked your interest in the bacterial genomics and molecular epidemiology of TB?

Philip Supply (PS): Early in my career, in Belgium, I studied very basic research questions related to yeast genetics. I wanted to change and conduct research on something that benefited the public directly. I contacted the head of a lab at the Institut Pasteur of Lille in northern France and he suggested that I work on TB. The Institut Pasteur of Lille is where the original anti-TB vaccine, the bacillus of Calmette-Guerin (BCG), was developed a century ago, so there's an extensive history of TB research here. During the conversation, I learned a lot about the contemporaneous extent of the TB problem.

Q: Why has TB been so difficult to eradicate?

PS: TB is a contagious disease that is silently transmitted. Patients can live without clear clinical signs of TB infection for quite some time before being diagnosed. This makes it very important to be able to properly diagnose the disease and trace its transmission. However, it is difficult to trace TB transmission in a population due to the properties of the bacterium, including its highly clonal population structure. The low diversity among TB strains results in the need for very precise molecular tools to trace strains and their spread in a population. The data that are obtained can be used to better identify routes of transmission among people with TB and all the people that they've been in contact with, as well as track the spread of epidemic strains to delineate outbreaks, in order to better control and prevent further propagation.

Q: How is TB treated currently?

PS: TB is very difficult to treat compared to other bacterial infections and requires a very demanding treatment regimen. The easiest course takes six months and works only in cases where the patient's TB strain is susceptible to current first-line antibiotics. In this regimen, the TB patient takes four drugs every day for two months. During the following four months, they need to continue to take two of those four drugs to ensure a cure.

Unfortunately, we have been using these first-line drugs for quite a long time now and resistant strains have developed. Patients afflicted with drug-resistant strains face a longer, even more complicated regimen. Treatment for these patients can take up to two years, with 5–7 different drugs that are less efficient and have more side effects. This has consequences for the treatment's rate of success and mortality. New treatment regimens that incorporate novel anti-TB drugs have a shorter course, but resistance to these new antibiotics, which begins to be seen for some of them, needs to be closely monitored and diagnosed as well.

The WHO estimates that approximately 450,000 new patients are suffering from rifampicin-resistant (RR) TB or MDR-TB each year.² However, only one in three of these patients are diagnosed as such and treated accordingly.² This diagnostic gap represents a huge problem.

Q: How do doctors know when they are dealing with a resistant strain of the disease?

PS: The classical way to identify a drug-resistant TB strain is to collect sputum from the infected patient, grow the specimen in culture, and test the obtained isolate for antibiotic susceptibility. If the strain is resistant to first-line TB drugs, like isoniazid and rifampicin, then additional cultures are done to test growth in the presence of second-line drugs. The difficulty with this approach is that these bacteria grow very slowly. It can take up to two months before a complete diagnosis can be made.

Q: Are there new drugs available to combat MDR-TB?

PS: A few new drugs have been released in the last few years, offering major progress in treating MDR-TB. Bedaquiline was approved in 2012, delamanid in 2014, and pretomanid in 2019 (in the US, 2020 in the EU). The WHO has recently announced that a six-month treatment regimen incorporating bedaquiline and pretomanid in combination with linezolid may be used in eligible patients with MDR/RR-TB, in place of longer treatments.⁴ Unfortunately, we are already seeing resistance emerge against some of these antibiotics. Resistance to these latest compounds must also be closely monitored and effectively detected.

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Q: How does the Deeplex Myc-TB assay identify drug-resistant strains of TB?

PS: Deeplex Myc-TB is a targeted NGS-based assay, in contrast to a whole-genome sequencing (WGS) assay. While a WGS assay could provide a comprehensive picture of all the mutations that occur in a TB strain, the large amount of DNA needed to run the assay is a problem. It can take between 7–10 days of TB culture to obtain adequate amounts of DNA for a WGS assay. This is a significant limitation that can delay identification of drug-resistant TB strains.

We moved to a targeted sequencing method where we first amplify the main drug resistance gene targets of the TB bacteria prior to sequencing. The Deeplex Myc-TB assay can be applied directly to DNA extracted from clinical samples with minimal bacterial loads, alleviating the need for culture and expediting analysis.

In addition to speed, another benefit of a targeted sequencing approach is depth of sequencing. Deep sequencing is important because it enables users to capture possible subpopulations of strains that carry drug-resistant mutations confidently. Even if these subpopulations represent a minority of the bacterial population, they can cause drug resistance because they will be selected under antibiotic pressure. The Deeplex Myc-TB assay can detect a bacterial subpopulation carrying a resistance mutation that represents as little as 1-3% of the sample.³

Another critical aspect of the success of the Deeplex Myc-TB assay is that we successfully developed a fully parameterized and automated web application for rapid, easy analysis and interpretation of the sequencing results. This makes the assay easily accessible to the many users who are not NGS specialists.

Q: How did you select the markers that you use in the Deeplex Myc-TB assay?

PS: We based our marker choices on our previous knowledge of the main targets of drug resistance in *M. tuberculosis*. We defined a marker set of 18 genes and included the known drug resistance mutations within those genes (Figure 1).

The assay can be upscaled easily. We are working to increase the number of markers to include targets for recently released drugs such as delamanid and pretomanid.

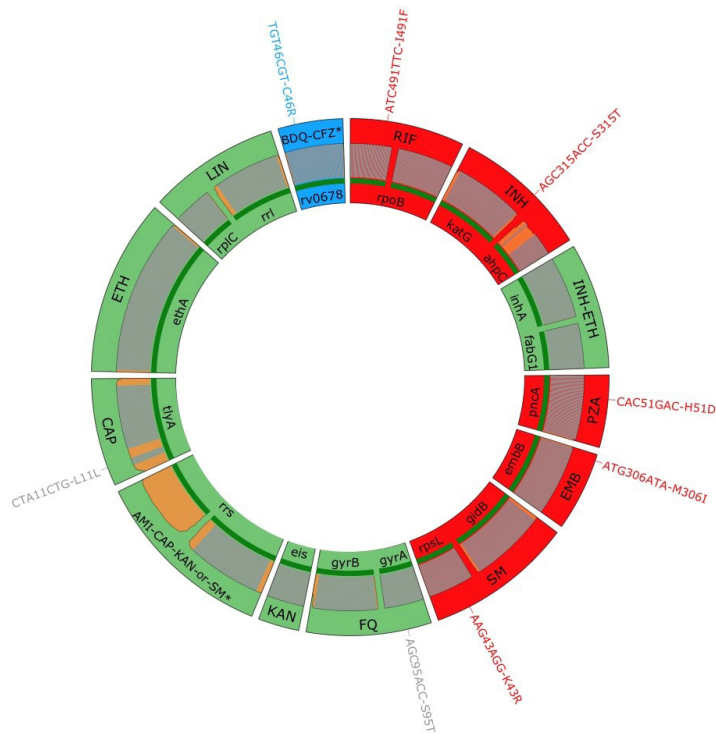


Figure 1: Deeplex Myc-TB Assay gene targets include markers for the detection of known (red) and potential (blue) anti-TB drug resistance-associated mutations.

Q: Has the Deeplex Myc-TB assay uncovered MDR-TB strains?

PS: We conducted a study of TB-positive cultures from four South African provinces where 1823 TB isolates had initially been identified only with isoniazid resistance using current molecular technologies, such as the GeneXpert MTB-RIF assay and line probe assays.⁵ We randomly selected 277 of those isolates and screened them with a rifampicin-resistant mutation-specific assay followed by the Deeplex Myc-TB assay and WGS using the MiSeq™ System to evaluate patterns of extensive resistance, transmission, and evolution. In 15% of the tested samples, we identified a special mutation that causes rifampicin resistance that is not captured in the current WHO-endorsed GeneXpert MTB/RIF molecular test or other tests. In addition, we identified mutations causing resistance to isoniazid and two other first-line drugs, thus reclassifying the isolates as MDR-TB strains resistant to all first-line antibiotics.

We also found that most of these strains were part of the same outbreak. As a result, these strains were escaping MDR surveil-

lance in the country. We also identified four distinct mutations in six isolates that are potentially associated with decreased bedaquiline sensitivity. It was completely unexpected. These patients were not supposed to have been treated with that drug, yet we saw probable signs of resistance emerging in that patient population.

"The Deeplex Myc-TB assay offers much more than the classical molecular methods...it amplifies > 10,000 base pairs, including the main TB drug resistance targets. It covers drug resistance—associated mutations for 13 anti-TB drug classes, comprising all those now defining extensively drug resistant TB (XDR-TB) according to the WHO."

Q: How could drug resistance to bedaquiline develop so quickly?

PS: It's a delicate question and, in this specific case, we don't have a definitive answer. Results from our phylogenetic analyses suggest that these mutations arose after the start of the clinical access program in the country for bedaquiline treatment of patients with MDR-TB. At the same time, we know that there can be cross resistance between bedaquiline and an earlier anti-TB drug, clofazimine. The main gene that is associated with bedaquiline resistance in clinical isolates is also the main gene that is associated with clofazimine resistance. Additional data are needed to better answer this question.

Q: How can the Deeplex Myc-TB assay identify mutations that older technologies cannot?

PS: The Deeplex Myc-TB assay offers much more than the classical molecular methods. It is more comprehensive because it amplifies > 10,000 base pairs, including the main TB drug resistance targets. It covers drug resistance—associated mutations for 13 anti-TB drug classes, comprising all those now defining extensively drug-resistant TB (XDR-TB) according to the WHO. In addition, our assay includes targets for *M. tuberculosis* genotyping, which is useful for molecular tracing, and detects more than 100 other mycobacterial species, comprising pathogenic or opportunist organisms of important medical relevance as well. Current technologies, including the GeneXpert MTB-RIF and line probe assays, can't match that.

The GeneXpert MTB-RIF assay is based on real-time PCR and it's fast, taking only two hours to produce a result. However, it covers only a limited region of the *rpoB* gene, which is a hotspot for mutations causing rifampicin resistance. That's why it missed identifying the new South African rifampicin-resistant strains. These strains carry a mutation that is outside this region in *rpoB*. The newer GeneXpert MTB-RIF XDR assay can detect certain resistance mutations to isoniazid, ethionamide, fluoroquinolones, and aminoglycosides, but does not provide information on other drugs, such as bedaquiline and linezolid representing now priority antibiotics for treating MDR-TB.

Line probe assays use PCR together with hybridization of online probes. These probes contain only a limited set of common resistance mutations for some, but not all, anti-TB drugs.

Representing another approach, the Oxford Nanopore system currently uses WGS, which captures more targets. As I indicated previously, a WGS assay requires a culturing step to obtain sufficient DNA amounts. Hence, the system is slow and the depth of coverage is usually lower. The level of sequencing accuracy, as far as we know, is not as high when compared to Illumina NGS systems. This is a significant limitation, especially for detecting mutations in minority populations. Available data indicate that the same limitation of accuracy with WGS applies to targeted sequencing on the Oxford Nanopore system.⁶

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Q: Are international organizations using the Deeplex Myc-TB assay?

PS: The WHO is using our assay for TB drug resistance surveys and it was mentioned in the organization's 2018 Technical Guide.⁷ WHO survey data have already been published, including several that involved data mining the levels of TB drug resistance in Djibouti, the Democratic Republic of the Congo, and Eritrea. Culturing is not performed systematically in these countries. As a result, the Deeplex Myc-TB assay was particularly useful because the assay could be run directly on clinical samples with no culturing required.

Q: Has the inclusion of the Deeplex Myc-TB assay in the WHO technical guide sparked an increase in health agencies using NGS assays for surveillance?

PS: It has increased the use of our assay for surveillance, as well as for rapid detection of TB drug resistance in individual patients. Surveillance can provide an accurate picture of TB epidemiology, that is the number of cases and prevalence of drug resistance in the total population, and an NGS-based assay can make a significant difference here. Both WGS and targeted NGS assays can provide extensive data on the prevalence of drug resistance in a patient population, based on genotypic information obtained from isolates (WGS) and specimens (targeted NGS) from individual patients.

As a targeted assay, Deeplex Myc-TB can also be applied in near real-time conditions as soon as a clinical sample is obtained. Such an assay could provide relevant information for rapid clinical decisions on the most appropriate treatment of a patient. In contrast, a WGS assay has the advantage of most precisely determining the genetic relatedness of isolates at a genome-wide

level. Therefore, it can also provide valuable information about the existence of outbreak strains in the population. As a result, targeted NGS and WGS assays are now used on an increasingly large scale by the WHO and many other health agencies.

"...Illumina NGS systems... are well known for offering high accuracy for detecting mutations. This is very important because we need to be able to detect even minority TB populations within a sample that are carrying drug resistance mutations."

Q: Why did you and the GenoScreen team choose Illumina NGS systems for the assay?

PS: We work with Illumina NGS systems, including the MiSeq, NextSeq™ 500, MiniSeq™, and iSeq™ 100 systems, because these platforms are well known for offering high accuracy for detecting mutations. This is very important because we need to be able to detect even minority TB populations within a sample that are carrying drug resistance mutations. The Illumina NGS systems are also the most used worldwide. If we develop the assay and validate it on these platforms, it gives us an important advantage because it will favor a more rapid and efficient roll out of the assay.

Q: Do you believe that the Deeplex Myc-TB assay will be useful in developing countries for TB surveillance and other kinds of medical work?

PS: We hope that the Deeplex Myc-TB assay will be a valuable tool in use throughout the world. GenoScreen and Illumina just engaged in a partnership to increase accessibility of this solution globally. As of today, the assay is being deployed in more than 35 countries on five continents, including in developing countries with high TB burdens and high MDR rates. An evaluation for clinical use is also being performed in cooperation with the Foundation for Innovative New Diagnostics (FIND). FIND obtained a grant from Unitaid specifically to test new targeted NGS-based tools for TB diagnostics in low- and middle-income countries.

Q: What challenges must be overcome for NGS-based TB assays to gain widespread use for TB surveillance and diagnostics?

PS: We'll need to make progress in developing the molecular biology infrastructure and skills in different countries. It might be easier to equip and train TB reference centers and labs that will use the NGS-based Deeplex Myc-TB assay than it would be to build capacity for bacterial culture-based assays. Indeed, the latter require special biosafety infrastructures that are not required for tests that can be directly applied on clinical samples. We are already organizing onsite trainings and videos that review the technical steps of the Deeplex Myc-TB assay workflow. This will ensure that the assay is well received and used most efficiently.

Another area of focus will be to train staff on assay data interpretation based on the detection of different mutations. To facilitate data interpretation, our web application for automated analysis of the sequencing data has a very user-friendly design. Finally, support and maintenance of the sequencing platforms needs also to be further improved for sustainable use in lower resource settings. All of these challenges will be addressed through the partnership between GenoScreen and Illumina.

Q: What are the next steps in the development of the Deeplex Myc-TB assay?

PS: In addition to adding new targets to the Deeplex Myc-TB assay to detect resistance to the latest TB antimicrobial drugs, we envision extending the assay to other mycobacteria. We now have an assay for *Mycobacterium leprae*, the mycobacterium that causes leprosy (Hansen's disease). Not unlike TB, many people think that leprosy is a disease of the past. While it is uncommon, it still exists in some parts of the world, including Africa, Brazil, India, and China. Similarly to Deeplex Myc-TB, Deeplex Myc-Lep detects drug resistance and identifies the strain genotype involved for use in diagnosis and surveillance. While it does not cover the complete genome, this targeted assay can differentiate strain types and drug-susceptible or drug-resistant strains that circulate in patient populations.

Learn more

MiSeq System, illumina.com/miseq

NextSeq 550 System, illumina.com/nextseq550

MiniSeq System, illumina.com/miniseq

iSeq 100 System, illumina.com/iseq

Deeplex Myc-TB assay, deeplex.com

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