

WHOLE-GENOME SEQUENCING IN RELATION TO RESISTANCE OF *MYCOBACTERIUM TUBERCULOSIS*

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Abstract

Tuberculosis, a disease caused by *Mycobacterium tuberculosis*, represents one of the deadliest infections worldwide. The incidence of resistant forms is increasing year by year; therefore, it is necessary to involve new methods for rapid diagnostics and treatment. One of the possible solutions is the use of whole-genome sequencing (WGS).

The WGS provides an identification of complete genome of the microorganism, including all genes responsible for resistance, in comparison with other genotypic methods (eg. Xpert MTB / RIF or Hain line-probes) that are capable to detect only basic genes. WGS data are available in 1-9 days and several online software tools (TBProfiler, CASTB, Mykrobe PredictorTB) are used for their interpretation and analysis, compared to 3-8 weeks in the case of classic phenotypic evaluation.

Furthermore, WGS predicts resistance to the first-line antituberculotics with a sensitivity of 85-100% and a specificity of 85-100%.

This review elucidates the importance and summarizes the current knowledge about the possible use of WGS in diagnosis and treatment of resistant forms of tuberculosis elucidates.

WGS of *M. tuberculosis* brings new possibilities for rapid and accurate diagnostics of resistant forms of tuberculosis. Introducing WGS into routine practice can help to reduce the spread of resistant forms of tuberculosis as well as to increase the success rate of the treatment, especially through an appropriate combination of antituberculotics ATs. Introduction of WGS into routine diagnostics can, in spite of the financial difficulty, significantly improve patient care.

Keywords: whole-genome sequencing, mycobacterium tuberculosis, drug resistance

INTRODUCTION

Mycobacterium (M.) tuberculosis is an acid-resistant intracellular bacterium which is known as causative agent of tuberculosis (TB). According to World Health Organisation (WHO) 1.7 milion people died from the disease in 2016 (including 400,000 among people with HIV) therefore nowadays tuberculosis is among the most common causes of death worldwide. However, even more disturbing is the emergence of drug resistance mostly to the first-line ATs, which significantly complicates the treatment. It is assumed that more than 50 million people are infected with resistant strains of *M. tuberculosis* worldwide. The most frequently diagnosed is *multi drug-resistant tuberculosis* (MDR-TB - 4% of all new cases), followed by *extensively drug-resistant tuberculosis* (XDR-TB) and during last years new cases of *totally drug-resistant tuberculosis* (TDR-TB) were detected (1). Despite the fact that incidence of tuberculosis in Slovakia is low, actual social changes in the community, cessation with vaccinations, as well as an increasing number of migrants may worsen the situation (Fig. 1).

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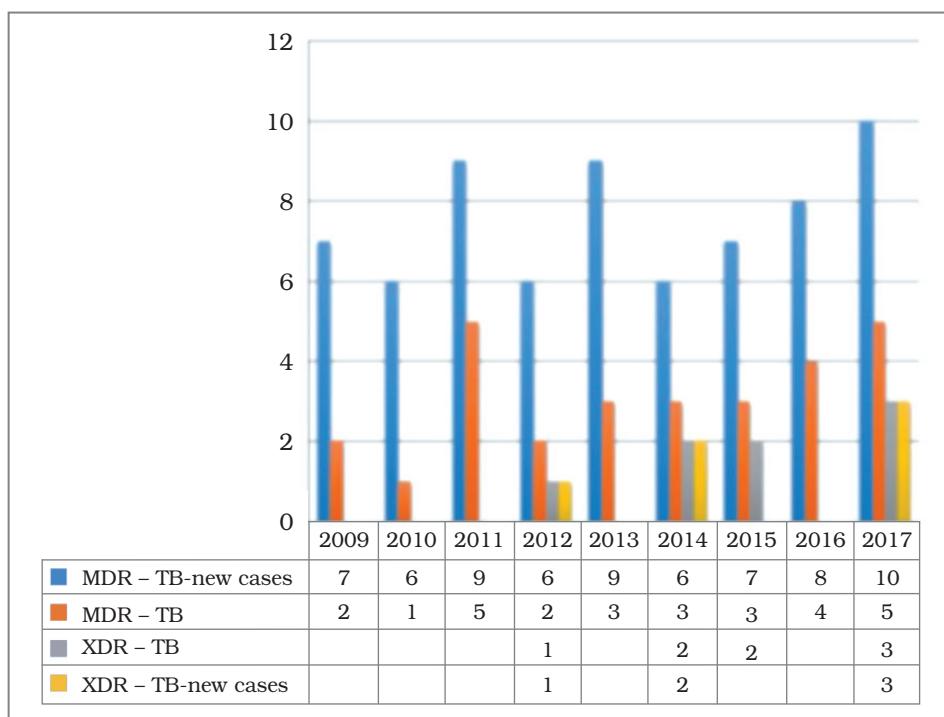


Fig. 1: The incidence of MDR and XDR – TB in Slovakia in the years of 1999-2017

Resistant TB represents a huge problem with a hopeless solution mainly in developing countries. For stopping this global threat an accurate and fast microbiological diagnostics along with the search for infected patients and their appropriate treatment is necessary. The diagnostics is based on an isolation of *M. tuberculosis* from biological material, identification of the strain, and determination of sensitivity to antituberculosis or direct determination of resistance to isoniazid, rifampicin, etambutol, aminoglycosides, cyclic peptides, and fluoroquinolones by molecular-genetic methods (2; 3).

According to WHO recommendations, the treatment of TB should include a combination of five effective drugs. However, it is not possible to achieve the effectiveness of the drug combination in many cases, especially because of the resistance or patient's intolerance to the drugs (4; 5). The overall success rate of TB treatment is 83%, which significantly reduces the incidence of resistant forms of bacteria. On the other hand, the treatment efficacy in MDR-TB patients is only 54%, whereas in patients treated for XDR-TB, the success rate is only 30% (6). Early diagnostics and the appropriate treatment may reduce spreading and development of resistant forms of TB; however, a low availability of fast diagnostic methods still generates a huge problem (7; 8). The WGS method is an alternative that could be used in diagnostic practice in near future as it overcomes the limitations of the currently used phenotypic and genotypic methods (9).

The goal of this review is to elucidate the importance and to summarize the current knowledge about the possible use of WGS in diagnostics and treatment of resistant forms of tuberculosis.

M. TUBERCULOSIS GENOME

The genome of *M. tuberculosis* (H37Rv strain) was completely sequenced for the first time in 1998. It contains approximately 4.4 million base pairs encoding 4,000 genes (most of which are involved in lipolysis and lipogenesis) and a high content of G+C pairs (65.6%) (10) (Fig. 2).

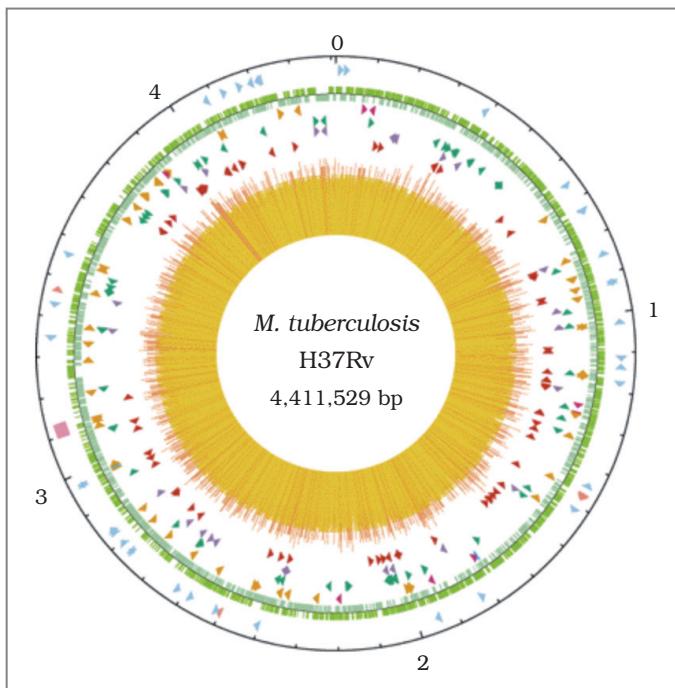


Fig. 2: Structure of *M. tuberculosis* genome (Cole *et al.*, 1998)

It has been assumed that the genetic variability between the individual strains is minimal and has no clinical significance. An important role in defeating this dogma played the WGS method, as it has been used for identification of a large number of strains. Furthermore, it has been confirmed that genomic variations are important in their phenotypic properties (e.g., increased virulence in the family of Beijing tribes) (11; 12; 13).

WHOLE-GENOME SEQUENCING (WGS)

The WGS method provides an identification of the complete DNA sequence (genome) of the organism at a particular time. It includes sequencing the chromosomal DNA, mitochondrial DNA, and, in the case of plants, also of DNA contained in chloroplasts. Technology development over the past 15 years has dramatically reduced the complexity, cost, and sequencing time. The mostly used sequencing platforms include IonTorrent, Illumina, and SMRT (14). The first step before the sequencing is a preparation of the sample. The DNA sample is split into fragments of the same length. The ends of the individual fragments are enzymatically edited to bind the respective adapters. Fragments with a length of 200-500 bp are selected for sequencing by the electrophoretic separation (these steps are so-called preparation of library). The last step before the sequencing is an amplification of DNA fragments which is usually done by the bridge polymer chain reaction (PCR). During the sequencing each of the four free deoxynucleotides (dNTPs) is labeled with a special fluorescent dye (Illumina – labeled dNTP). After incorporation of the nucleotides the fluorescent signal is measured. The process is repeated until the entire library is captured (15; 16) (Fig. 3).

WGS has a great potential to become an inevitable tool in controlling the antibiotic resistance, the major threat in modern healthcare. WGS has already reached a number of applications in this area. These include the development of new antibiotics, diagnostic tests, or the control of antibiotics by monitoring and clarifying factors that influence the onset and persistence of resistance (17). The portable Minion sequencer (Oxford Nanopore Technologies, United Kingdom) has been extensively used in the diagnosis of Ebola virus cases in West

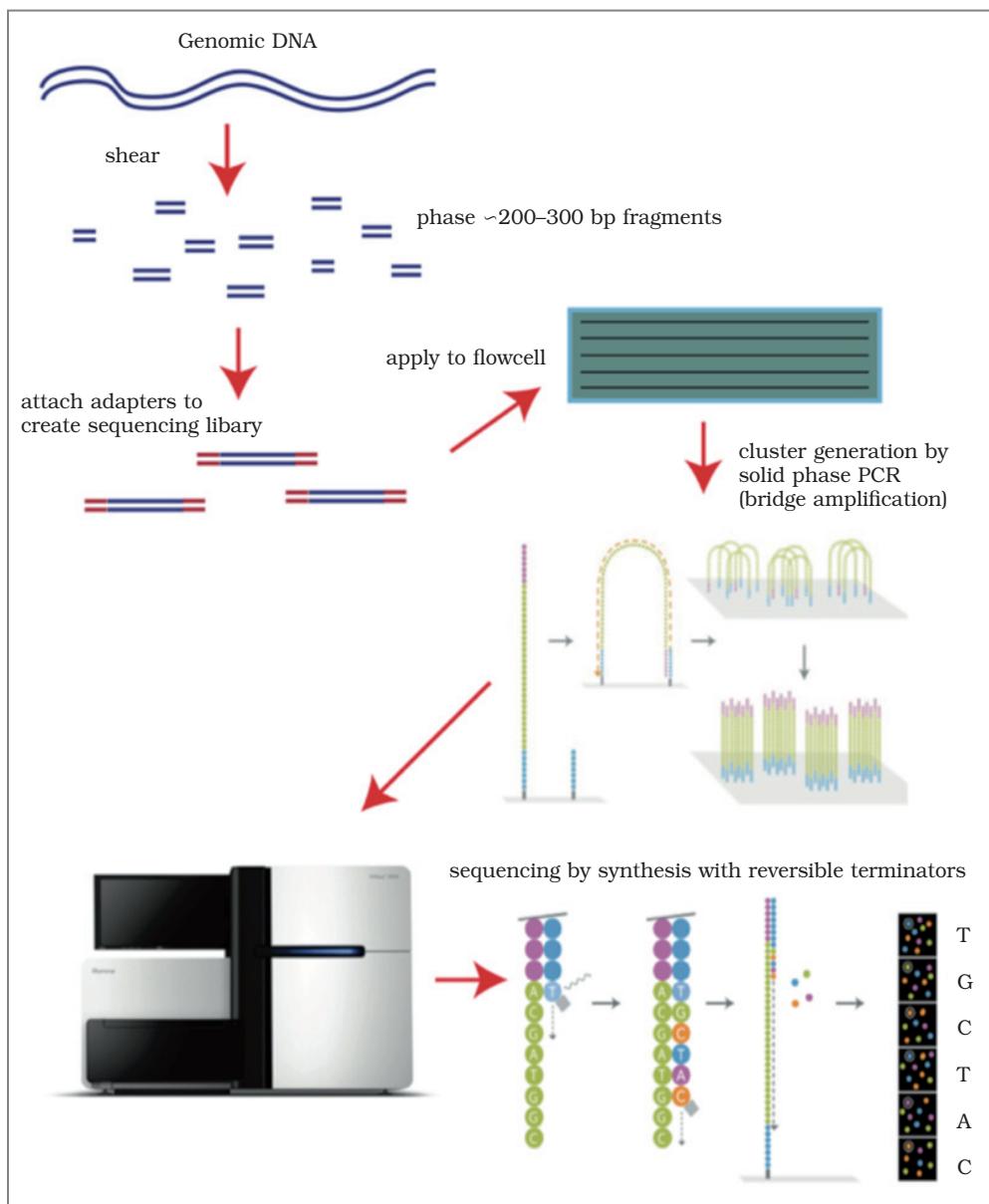


Fig. 3: WGS on Illumina Miseq (source: <https://bitesizebio.com/13546/sequencing-by-synthesis-explaining-the-illumina-sequencing-technology/>)

Africa and the advantage of this machine lies in its ability to identify pathogens and antimicrobial resistance directly from urine (18; 19). Based on the WHO guidelines a network of testing centers and special laboratories has been set up for using WGS to determine HIV virus resistance against anti-retroviral drugs (20).

WGS AND *M. TUBERCULOSIS* RESISTANCE

In addition to clinical use in the field of cancer and hereditary diseases, WGS may lead to a breakthrough in the diagnosis of infections caused by *M. tuberculosis*. It enables the iden-

tification and detection of the susceptibility of the test strain in a clinically relevant time-frame, contrary to conventionally used methods based on phenotypic examination (this may take up to 8 weeks) (21; 22) (Fig. 4).

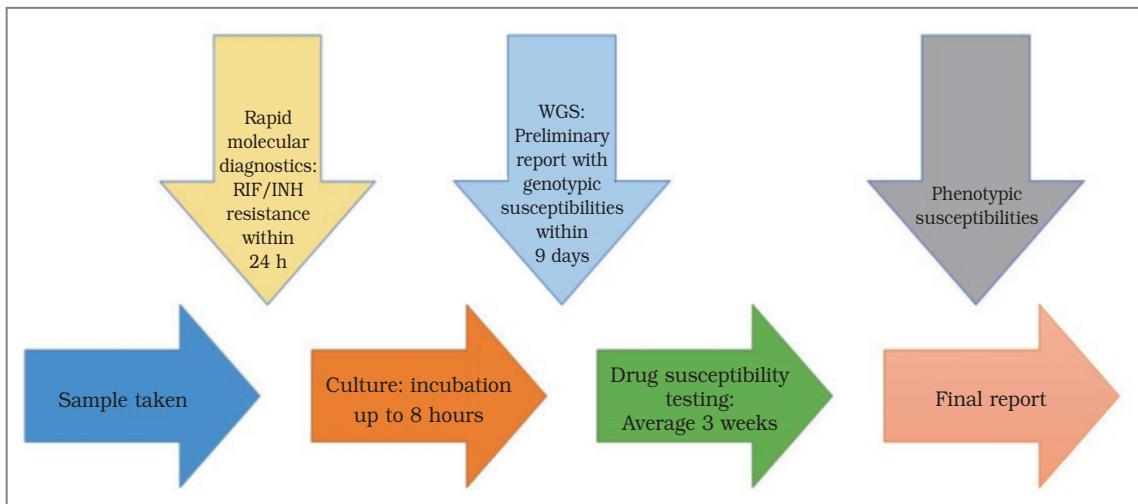


Fig. 4: Comparison of evaluation by genotypic (Xpert, WGS) and phenotypic methods. Results of genotypic methods are available in 1–9 days. In the case of classical phenotypic evaluation, it is 3–8 weeks (according to Satta et al., 2017).

M. tuberculosis resistance is induced due to a large extent single-nucleotide polymorphism (SNP), insertions, deletions, and to a significantly lesser extent to horizontal gene transfer via plasmids or transposons (23). SNPs represent all single nucleotide variants in the *M. tuberculosis* genome when compared to the reference genome, and mutations mean a subset of the SNPs that are relevant or functional concerning the resistance (24). It is assumed that changes in the regulation of efflux pumps also play a role in the development of resistance (25). Currently, there is a number of well described gene mutations and the mechanisms of resistance caused by these mutations (Tab. 1).

The standardized methodology for interpretation of the relationship between mutations and the phenotypic signs of resistance to ATs has been developed recently. Mutations are divided into several groups with a high, intermediate, minimal, or indefinite relationship to resistance or are not associated with resistance at all (26). However, the mechanism of resistance is not known in 10–40% of resistant strains. It is supposed that SNP are responsible for resistance in most cases.

WGS is used for accurate identification of characterized and non-characterized mutations and significantly supports the continuous development of a reference library of mutations responsible for resistance (e.g. online software "TB profiler") (27; 28). Recently, WGS has confirmed that a frequency of new mutations is higher in the TB positive patients with failing treatment compared to the successfully treated patients (29; 30). In addition, WGS has also discovered that some mutations previously considered as markers of resistance to etambutol are in fact only markers for a particular phylogenetic *M. tuberculosis* family. For this reason false positive results have often occurred in countries with an occurrence of this phylogenetic family and this knowledge should be considered in developing new genotypic methods determining the sensitivity of bacteria (17).

Progress has been made in interpreting and standardizing the data. There are several online software tools providing a quick interpretation of WGS data. Online databases (ReSeqTB, TB Portal, GenTB) serve as an option to share, interpret, and link genetic results

Tab. 1: Overview of genes encoding resistance and mechanisms of *M. tuberculosis* resistance (Bang, 2010)

Drug	Gene	Function of the gene	Mechanism of resistance
Rifampin	<i>rpoB</i>	Beta subunit of RNA polymerase	Inhibition of RNA synthesis
Isoniazid	<i>katG</i>	Catalase-peroxidase	Reduces of ability to activate the prodrug isoniazid
	<i>inhA</i>	Enoyl ACP reductase	Reduces the binding of NADH to inhA and attack by isoniazid
	<i>ahpC</i>	Alkyl hydroperoxide reductase	Overexpression of antioxidant enzyme AphC, that removes peroxide necessary for isoniazid activation
	<i>kasA</i>	Beta-ketoacyl ACP synthase	Overexpression of KasA, involved in fatty acid and mycolic acid synthesis
	<i>ndh</i>	NADH dehydrogenase	Increases the NADH/NAD ratio, competes with the binding of isoniazid-NAD to inhA
Ethambutol	<i>embB</i>	Arabinosyl transferase	Decreases the binding to ethambutol
Pyrazinamide	<i>pncA</i>	Pyrazinamidase	Lack of conversion of pyrazinamide to pyrazinoic acid
Streptomycin	<i>rpsL</i>	S12 ribosomal protein	Decreased binding of streptomycin to S12 ribosomal protein
Amikacin	<i>rrs</i>	16S rRNA	Decreased binding of aminoglycosides to 16S rRNA
Kanamycin	<i>rrs</i>	16S rRNA	Decreased binding of aminoglycosides to 16S rRNA
Capreomycin	<i>tlyA</i>	2'-O-metyltransferase	
Fluoroquinolones	<i>gyrA</i> , <i>gyrB</i>	DNA gyrase	Inhibition of DNA gyrase
Ethionamide	<i>inhA</i>	Enoyl ACP reductase	Inhibition of mycolic acid synthesis

ACP = acyl carrier protein, NADH = nicotineamid adenine dinucleotide, reduced form

with the phenotype of drug resistance and other epidemiological data. These and other databases also allow control of *M. tuberculosis* resistance at the global level (31).

COMPARISON OF WGS WITH OTHER MOLECULAR-GENETIC METHODS

Currently used genotyping methods (eg. Xpert MTB / RIF or Hain line-probes) allow in a relatively short time the identification of individual species of mycobacteria and mutations responsible for their resistance (32). However, the number of identified mutations is very limited. This limitations show major disadvantages compared to WGS (33; 34; 35; 36; 37).

Xpert / RIF is a genotypic method using PCR to identify *M. tuberculosis* and to determine *rpoB* gene mutations and to confirm a resistance to rifampicin. As soon as positive results

are obtained the treatment of MDR-TB (caused by a strain resistant to rifampicin and isoniazid) is initiated. However, this procedure is often incorrect. In countries like Iran and India up to 33% of rifampicin-resistant strains do not exhibit resistance to isoniazid and such treatment may lead to the development and spread of MDR-TB (38; 39). For this reason, in addition to the Xpert MTB / RIF, the phenotypic evidence of sensitivity must also be obtained in the US (40). A suitable solution may be the use of WGS method as it allows the identification of a number of genes encoding resistance at the same time, thereby leading to the appropriate treatment.

Coll et al. evaluated isolates from different countries of the world to obtain information about sensitivity and specificity of WGS in resistance prediction compared to phenotypic methods. High values of sensitivity and specificity were confirmed for first-line ATs: rifampicin 96.2% and 98.1%, isoniazid 92.8% and 100%, ethambutol 88.7% and 81.7%, and streptomycin 87.1% and 89.7% (33). In a study published by Walker et al. the authors showed the following sensitivity and selectivity values: rifampicin 91.7% and 99.2%, isoniazid 85.2% and 98.4%, ethambutol 82.3% and 95.1%, and streptomycin 86.1% and 99.1% (41). These results were later verified by a research team from India confirming 100% sensitivity and 94% specificity for rifampicin and isoniazid, 100% sensitivity and 78% specificity for ethambutol, and 85% sensitivity and 100% specificity for streptomycin (42). Shea et al. decided to use WGS for all clinically confirmed cases of tuberculosis in New York. Species identification was determined with an accuracy of 99%. The compliance rate of resistance determined by WGS compared to culture sensitivity tests was 96%. In addition, the doctors received results with resistance profiles 9 days earlier for the first-line antituberculosis and up to 30 days earlier for the second-line antituberculosis (ethionamide, kanamycin, ofloxacin) (43).

OTHER USE OF WGS

The greatest potential of WGS for the clinical practise (but also the biggest challenge) is the ability to sequence the individual strains of *M. tuberculosis* directly from clinical isolates without the cultivation. Doughty et al. diagnosed 7 of 8 positive samples from patients. Although the identification of the *M. tuberculosis* species has provided useful information, a large number of non-tuberculous sequences in combination with insufficient depth of coverage (number of reads covering the particular target nucleotide) and depth of sequencing (total amount of sequencing data) make it impossible to predict resistance (44). Therefore, it is necessary to get the highest possible concentration of *M. tuberculosis* DNA in the sputum for the direct evidence of resistance. For this purpose there are designed special RNA templates (RNA baits, SureSelectXT, Agilent, US) (45). The use of these templates led to confirmation of resistance to rifampicin and isoniazid with 95–97% sensitivity and 98–100% specificity in 96 hours directly from sputum (46; 47).

Tuberculosis caused by resistant strains does not arise only from selective pressure occurring due to inappropriate therapy or at sub-therapeutic doses of the used ATs. XDR-TB often develops after several episodes of ineffective treatment, including the use of second-line AT for treating MDR-TB. However, it is important to note that tuberculosis induced by MDR and XDR strains may also be caused by direct infection by resistant strains from the infected patients (48). WGS allows to study the origin and transmission of resistant forms, particularly in high burden TB countries (49).

Historically it was believed that patients were infected with only one strain of *M. tuberculosis*. This assumption was rebutted by the development of molecular epidemiology technology. WGS has confirmed that TB is a disease caused in many cases by a complex of different strains (50).

In addition to the above-mentioned possibilities of usage, the WGS is a tool for identifying target sites of AT (e.g. bedaquilin drug target) and for patients with recurrent infections to determine whether it is a relapse (patient infected with the same strain) or reinfection (patient infected with a new strain) (51; 52).

CONCLUSIONS

Whole genome sequencing of *M. tuberculosis* brings new possibilities for rapid and accurate diagnostics of resistant forms of tuberculosis. Introducing WGS into routine practice can help to reduce the spread of resistant forms of tuberculosis as well as to increase the success rate of the treatment, especially through an appropriate combination of ATs. The introduction of WGS into routine diagnostics can, in spite of the financial difficulty, significantly improve patient care.

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