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A Review of Phenotypic and Genotypic Methods for Detection of Drug Resistance in *Mycobacterium tuberculosis*

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ABSTRACT

Tuberculosis is one of the most dangerous infectious diseases in the world and causes nearly two million deaths each year, especially in developing countries. Meanwhile, multidrug resistance tuberculosis (MDR-TB) is due to the resistance of *Mycobacterium tuberculosis* (Mtb) strains to two effective first-line drugs, isoniazid and rifampin, which is increasing worldwide. MDR-TB strains are mainly caused by inadequate treatment of TB patients. The emergence and spread of these strains is an obstacle to the control and management of tuberculosis as well as a threat to the World Health Organization's goal of eliminating the disease by 2050. Proper management of MDR-TB relies on early recognition of the disease. Recently, phenotypic and genotypic diagnostic methods have been developed to rapidly identify MDR strains in tuberculosis patients. Some of them are also economically suitable to use in developing countries. Proper treatment of patients with drug-resistant TB requires the rapid detection of resistant strains and appropriate drug administration. Regular monitoring of patients' side effects of medications as well as enhancing the quality of bacterial tests is essential to identify resistant strains. Therefore, in this review, we will describe the available phenotypic and genotypic tests for drug-resistant tuberculosis detection and discuss their advantages and limitations.

Keywords: Mycobacterium tuberculosis, Drug resistance, MDR-TB, Drug susceptibility test

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Introduction

Tuberculosis has been one of the deadliest infectious diseases in the world and remains a public health threat **(1)**. In 2018, the World Health Organization reported more than 10 million (between 9 and 11.1 million) new cases of tuberculosis **(2)**. The emergence of multidrug-resistant Mycobacterium strains is increasing due to various factors such as AIDS epidemic, irregular use of anti-TB drugs, increased use of injectable drugs and migration from endemic areas **(3)**. Multidrug Resistance Tuberculosis (MDR-TB) is defined as resistance to two effective first-line drugs for the treatment of tuberculosis, isoniazid and rifampin **(4)**. MDR-TB strains with extensive drug resistance (XDR-TB) are described as resistance to all oral fluoroquinolones and at least one second-line injectable aminoglycoside (amikacin, caproomycin and kanamycin) **(5)**.

In 2018, the World Health Organization estimates that %3.4 of new cases and %18 of previously treated MDR-TB cases, as well as %8.5 of MDR-TB cases associated with XDR-TB **(2)**. In order to prevent the spread and spread of MDR-TB and XDR-TB strains and

the emergence of new strains, simple, rapid and accurate diagnostic methods are needed to identify drug resistance among patients with tuberculosis. Unlike many bacteria where antibiotic resistance occurs due to motile genetic elements such as plasmids, transposons and integrons, mycobacteria have a chromosomal drug resistance and are often caused by mutations associated with a limited region of the genome. This resistance can be transmitted to subsequent generations of bacteria and disrupt TB control and treatment programs **(6, 7)**. In the following, the mechanisms of action and resistance of isoniazid and rifampin drugs as well as the advantages and limitations of TB drug sensitivity diagnostic tools and methods are presented to evaluate and evaluate the advantages and disadvantages of each of these methods.

Conventional phenotypic methods on solid medium

The most commonly used egg-based culture media is the Levenstein-Johnson (LJ) medium.

Proportional method

In this method, growth rate of Mtb on the control medium without antibiotic is compared to the growth on the drug-containing medium to determine susceptibility or resistance. The number of colonies counted in the control tube indicates the number of live bacilli in the total number of microbes cultured and the number of colonies in the antibiotic-containing tube indicates the number of resistant bacilli in the same number of microbes. The ratio of the first number to the second number is called the critical ratio or percentage of resistance, indicating that the strain is resistant or sensitive. For different drugs the criterion of resistance (critical ratio) varies. For example, the percent resistance for both isoniazid and rifampin is 1% **(26, 27)**.

Absolute Concentration Method

In the absolute concentration method, a standard amount of bacteria is inoculated in a solid medium such as LJ containing different concentrations of the antibiotic. The lowest drug concentration that inhibits bacterial growth (less than 20 colonies in 4 hours) is defined as resistance criteria. **(28)**.

Resistance Ratio Method

This method is similar to the absolute concentration method and is the ratio of the drug MIC for the tested strain to the MIC for the standard H37RV strain performed under the same conditions **(29)**.

Conventional Phenotypic Methods in Liquid Medium

Using liquid media instead of solid media reduces the cultivation time from 8-12 weeks to 3-7 weeks. These environments can also be stored for longer periods at room temperature **(30)**.

BACTEC 460 TB SYSTEM Radiometric Method

The culture medium used in the radiometric method is 7H12 (12A) medium. Mtb metabolize palmitic acid containing radioactive carbon in this medium and release CO_2-labeled gas in the upper part of the culture medium **(31)**. This gas is collected and measured by a semi-automatic device called the BACTEC 460 TB SYSTEM. Determining the amount of radioactive carbon in CO_2, the growth rate of Mtb is accurately determined. This growth rate is called the GI index, which indicates a positive mycobacterial culture if it is 10 or more **(32)**.

Mycobacterial Growth Indicator Tube (MGIT) Method

This method uses modified Middlebrook 7H9 with a fluorescent extinguishing oxygen sensor mounted at the end of the tube. In addition to the compounds in the 7H9 environment, it contains a mixture of antimicrobial agents such as PANTA (polymyxin, amphotericin, nalidixic acid, trimethoprim and azlocillin) to prevent the growth of gram-positive and gram-negative bacteria. The consumption of oxygen in the fluorescence medium and the detection of this light in the presence of UV lamps is a reason for the growth of Mtb in the tube **(34)**.

Versa TREK Method

This method uses modified Middlebrook 7H9 with a mixture of antimicrobial agents such as PVNA (polymyxin, vancomycin, nalidixic acid, and azlocillin). This method is capable of simultaneously identifying mycobacterial growth and drug sensitivity to first-line drugs by measuring changes in oxygen consumption **(35-36)**.

Modern Phenotypic Methods

These methods include mycobacteriophage expressing luciferase, colorimetric methods, and nitrate reduction test.

Genotypic Methods for the detection of MDR-TB resistant strains

Molecular methods are capable of detecting genes that are effective in generating drug resistance and resistance-related mutations in Mtb target genes. Using these methods, the results are obtained within 1 to 2 days and can be directly applied to smear positive sputum isolates and other clinical specimens.

Amplification Refractory Mutation System (ARMS)

ARMS-PCR is a simple and rapid method for identifying point mutations, restriction fragment length polymorphisms (RFLPs) or small deletions during DNA fragment sequencing (46). This method is

also called allele-specific PCR or PCR amplification of specific alleles (PASA).

DNA-Sequencing Method

DNA sequencing is the most widely used genotypic method for detecting drug resistance, especially first-line TB drugs in Mtb. Sequencing is the most accurate and reliable method for mutation detection and is used as the gold standard method **(49)**.

PCR-Single Stranded Conformation Polymorphism Analysis (SSCP)

PCR-SSCP is a simple and rapid method that can be used to determine the presence or absence of a mutation in a specific region of DNA based on the pattern of DNA migration in the gel. As a rapid screening tool, it has high accuracy in the detection of drug resistance, especially in MDR-TB cases **(51)**.

PCR-restriction Fragment Length Polymorphism

The PCR-RFLP method is a simple, rapid, and inexpensive method used to detect changes in one or more codons found in drug resistant and mutation sequences **(53)**.

Determination of Antibiotic Susceptibility by Real Time PCR

The Real Time PCR method is very similar to the conventional PCR method. Similar to PCR, a sequence is amplified using specific primers. But Real Time PCR differs from conventional PCR in quantitative amplification of sequences. In Real Time PCR, the amplification of the product is detected by using the fluorescent marker in the reaction. These fluorescent markers are designed to produce light by binding to DNA if they replicate. The Real Time PCR method is divided into two categories: (1) the use of nonspecific fluorescent markers using DNA-bound dyes such as SYBR® Green or Eva green (2) the use of dedicated fluorescent markers using probes Target genes.

High Resolution Melt-HRM Real Time PCR

This method first amplifies the resistance-related genes by Real Time PCR and then the PCR product is heated in the presence of specific DNA fluorescent dyes such as SYBR-Green and Eva-Green. Colours are specific for double stranded DNA. At the beginning of the rise in temperature, the signal is high because at low temperatures most DNAs are double stranded. As the temperature continues to rise, DNA begins to separate and single-strand and thus loses colour. **(62)** In the HRM method, differences between different genotypes are determined by differences in the melting curve. That is, even a single change in gene sequence (mutation) can affect Tm and cause the fragment's melting curve to change.

Determination of Antibiotic Susceptibility by Line Probe Assay (LiPA)

Line probe assay (DNA probe assay) is a method based on DNA Strip Test, which involves DNA extraction, amplification of a gene associated with resistance, and subsequent hybridization of PCR products labelled with oligonucleotide probes fixed on the strip. These oligonucleotides are highly sensitive and do not bind to complementary DNA if they contain even one different nucleotide.

INNO-LiPA Rif. TB (Innogenetics)

This method searches for rifampin resistance mutations in the *rpoB* gene. Since rifampicin resistance is an indicator for the detection of MDR-TB, positive results of this method detect about 90% of MDR-TB samples **(68)**.

Xpret MTB/RIF (cephied) Method

The Xpret MTB/RIF method is able to simultaneously detect Mtb complex and its resistance to rifampin antibiotics directly from sputum collected within two hours. It should be noted that a high proportion of rifampin-resistant strains are associated with concomitant resistance to isoniazid (approximately 95%) and individual resistance to rifampin accounts for only about 5% of the resistant strains. Therefore, rifampicin resistance can be used as a high-accuracy MDR-TB index.

Discussion

Among the phenotypic methods available, the proportional method of drug resistance testing has a high sensitivity and specificity compared to other methods. But the only problem with the relative method is the relatively long time required to report the results **(26, 27)**. Therefore, the use of molecular methods is very helpful in reaching a faster report. Among the molecular methods available, the GeneXpret MTB/RIF method is able to simultaneously identify the Mtb complex and its resistance to rifampin antibiotics directly from sputum collected within two hours. The accuracy, sensitivity, and specificity of this method are acceptable. The only drawback to using this method is the dependence on special cartridges that are necessarily imported from abroad **(76-74)**. Therefore, it is recommended to launch another suitable molecular method with appropriate accuracy such as drug resistance evaluation using TaqMan Real Time PCR.

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Conflict of Interest

Authors declared no conflict of interests.