Current approaches in drug-resistant tuberculosis diagnosis

Actualități în diagnosticul tuberculozei drog-rezistente

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Abstract

Drug-resistant tuberculosis (DR-TB) increases mortality and threatens the progress achieved in the management of TB, due to late diagnosis and ineffective treatment. Thus, the accurate identification of this disease becomes a priority, with important therapeutic and public health implications. New molecular techniques ensure a more rapid diagnosis of DR-TB, and can have a substantial impact on the disease prognosis, but there is still the need for inexpensive diagnostic tests available for all patients. The objective of this review is to give an overview on the new developments made in DR-TB diagnosis, based on a selective research of literature reports and World Health Organization guidelines.

Keywords: *MDR-TB, XDR-TB, LPA, Xpert® MTB/RIF*

Rezumat

Tuberculoza drog-rezistentă (TB-DR) crește mortalitatea și amenință progresele înregistrate în diagnosticul TB, ca urmare a depistării tardive și ineficienței tratamentului. În acest context, identificarea corectă a acestor infecții devine o prioritate, cu implicații importante asupra conduitei terapeutice și a sănătății publice. Tehnicile de biologie moleculară nou apărute asigură un diagnostic mai rapid al TB-DR, cu impact semnificativ asupra evoluției bolii, dar rămâne în continuare problema dezvoltării unor teste de diagnostic rapide și necostisitoare, accesibile tuturor pacienților. În acest articol ne propunem să oferim o scurtă trecere în revistă a progreselor realizate în diagnosticul DR-TB, bazată pe datele publicate în articolele de specialitate și rapoartele Organizației Mondiale a Sănătății.

Cuvinte-cheie: *TB-DR, XDR-TB, LPA, Xpert® MTB/RIF*

Introduction

Tuberculosis (TB) remains a major global health problem, causing 1.4 million deaths in 2015[1], despite the global efforts to reduce the burden of this disease. In the last fifteen years, the TB incidence decreased with a rate of 1.4% per year[1], but the emergence of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) poses a new threat to the control and management of TB[1-5]. These forms of TB have increased mortality, lower cure rates[6,7,8], and require longer treatment regimens (at least 20 months) compared to drug-susceptible TB[6,9]. Thus, in areas where TB is endemic, new routine diagnostic tests that identify MDR and XDR strains are needed for prompt diagnosis and treatment[6]. According to World Health Organization (WHO) report from 2013, MDR-TB was detected in all surveyed countries, while at least one case of XDR-TB was identified in 84 countries[6,9]. The global burden of MDR-TB and rifampicin-resistant TB (RR-TB) in 2015 was estimated to be 3.9% of the new cases and 21% of the previously treated TB cases, respectively[1], with China, India and the Russian Federation accounting for nearly half of these cases (45%)[1]. For the same year, in WHO European Region, the estimated incidence of MDR/RR-TB was 16% of new cases and 48% of previously treated cases of TB, most of these belonging to Eastern European countries[1]. As a result, WHO's Regional Office for Europe adopted the Tuberculosis action plan for the WHO European Region 2016-2020[10]. This plan sustains the early diagnosis of all forms of tuberculosis and universal access to drug-susceptibility testing (DST), including the use of rapid tests[11].

Objective

The objective of this review is to give an overview on the new developments in DR-TB diagnosis, based on a selection of literature reports and WHO guidelines and to summarize the strategy used for prevention, surveillance and control of DR-TB in our country.

Definitions

Mycobacterium tuberculosis strains resistant to both first-line drugs, isoniazid (INH) and rifampicin (RIF) are defined as MDR-TB[2,6,12]. XDR-TB strains were first reported in March 2006, in relation to a severe form of disease caused by strains with resistance to INH, RIF and at least three second-line drugs. Because DST is more reliable for fluoroquinolones (FQ) and injectable drugs than for other second-line drugs, in October 2006 the definition was revised as resistance to at least INH and RIF and, in addition, to any FQ and to at least one of the three injectable drugs – capreomycin (CAP), kanamycin (KAN) and amikacin (AK)[2,3,6].

Laboratory diagnosis of MDR-TB and XDR-TB

Rapid determination of drug resistance and early choice of adequate antibiotic determine the outcome of disease, thus the laboratory is an essential component in TB control[2,13]. Inappropriate choice of therapy, caused by delayed identification of drug resistance, may generate additional drug resistance, continued transmission in the community or may result in death within weeks, as in the XDR-TB in HIV-infected patients[2]. DR-TB diagnosis is difficult because DST has not become a routine test in many national surveillance TB programs[2]. Globally, in 2015, DST for RIF was performed only in 24% of new TB cases and 53% of previously treated TB cases, while DST for FQ and second-line injectable drugs was conducted only in 36% of notified MDR/RR-TB cases[1]. The absence of affordable rapid and accurate diagnostic techniques for drug resistance applicable in high-incidence areas makes the diagnosis of MDR- and XDR-TB more difficult[2].

Conventional culture-based methods

For more than a century, particularly in countries with endemic TB, sputum smear microscopy was the basis for diagnosis, in spite of its low sensitivity (~40% compared with culture) and its incapacity to indicate antibiotic susceptibility[6]. This method was later supplemented by culture in developed countries or higher level hospitals in developing countries[6]. However, culture requires specialized biosafety facilities that limit its use. Also, as a result of the slow growth rate, diagnosis of drug resistance can take at least 1-2 weeks, and as much as 1-3 months[6,14]. Phenotypic DST methods (agar proportion, absolute concentration and resistance ratio) assess inhibition of *M. tuberculosis* growth in the presence of antibiotics and may require 8 to 12 weeks to identify DR-TB on solid media such as Lowenstein-Jensen[2]. However, despite being a time-consuming technique, conventional DST remains the gold standard in the diagnosis of DR-TB[15].

Automated liquid culture systems

Automated liquid culture systems have higher sensitivity for the detection of resistance to first- and second-line anti-TB drugs, but still, 2 to 4 weeks are needed for results, and their use is constrained by high cost[2]. They are based on detection of mycobacterial growth in the presence of anti-TB drugs by radiometric (BACTEC 460 TB system, Becton Dickinson, USA), fluorescent (BACTEC Mycobacterial Growth Indicator Tube-MGIT 960, Becton Dickinson, USA), colorimetric (MB/BacT system, Organon Teknika, The Netherlands) or oxygen consumption measurements (Thermo Scientific™ Versa TREK system)[15,16]. DST on liquid culture represents the standard diagnosis in developed countries. In 2007, WHO recommended the use of automated liquid culture systems to improve diagnosis of MDR-TB in low and medium income settings and offered advising on financial support, infrastructure, human resources, costumer support, specimen collection, recording and reporting results[17].

New diagnostics for drug-resistant TB

New rapid phenotypic methods are represented by microscopic-observation drug-susceptibility test (MODS), thin-layer agar technique, colorimetric methods, nitrate reductase assay and phage amplification-based test. MODS technique is a low cost direct assay that detects *M. tuberculosis* specific cording growth in liquid medium supplemented with first line anti-TB[2,18,19]. This technique is applicable even for sputum samples with negative microscopic examination[20] and has a 92-96% sensitivity and a specificity of 96%[15]. The results are obtained within 2-4 weeks but require daily microscopic examination[19]. Thin-layer agar technique detects mycobacterial growth on the surface of the Middlebrook agar supplemented with RIF and HIN using conventional microscopy. The results are obtained in 11 days from sputum samples, with a 100% sensitivity and specificity[21]. The colorimetric methods such as microplate Alamar Blue assay, resazurin microtitre assay and tetrazolium microplate assay show mycobacterial growth using redox reactions when pure culture broth is inoculated in a concentration gradient of anti-TB drugs in the microplate containing certain dyes (Alamar Blue solution, rezasurin, tetrazolium bromide)[19]. These methods have good sensitivity and specificity and provide results in 1-2 weeks, being accessible to laboratories with limited resources[2,22]. The detection of MDR-TB in the smear-positive sputum sample with the nitrate reductase assay is another rapid (10 days) and low cost method, based on reduction of nitrate to nitrite in the presence of bacterial growth[19]. The techniques using bacteriophages detect *M. tuberculosis* resistant to RIF, either by the phage amplification method, or by luciferase use. The results are obtained in 48-72 hours. Test's sensitivity is higher in culture (95%) than in sputum[23]. The above mentioned rapid phenotypic methods require trained personnel, quality control standards and further evaluation of their accuracy for sensitivity testing to second-line anti-TB drugs[2].

Ideally, **new and molecular methods** for diagnostic of DR-TB should be rapid, inexpensive, and thus, available for patients in low income settings and should require no specialized training or facilities[6]. Novel diagnostic tests have some, but not all of these characteristics[6] and are based on identification of specific mutations that induce resistance to anti-TB drugs[2]. For 96% of RIF-resistant strains, the site of mutation is the 81 bp core region located between codons 507 and 533 of *rpoB* gene[24], encoding for the β subunit of RNA polymerase[6,25]. Most commonly affected are the codons 531, 526 and 516, mutations in these sites causing high-level resistance to RIF and cross-resistance to other rifamycins[24,26]. RIF resistance is considered as a "surrogate" marker for the diagnosis of MDR-TB because in 90% of RIF-resistant strains it is preceded by INH resistance and monoresistance to RIF is rare[19,26]. For INH, although the target is *inhA*, a protein involved in mycolic acid synthesis[6,27], high level resistance is more often caused by mutations in *katG,* between codons 138 and 328, encoding a mycobacterial catalase, required for activation of the pro-drug INH[6,28]. The codon 315 of *katG* is the most commonly affected (50-90%) [24,29]. The promoter region *mabA-inhA* of the gene *inhA* is the second most frequently affected by mutations, especially in position -15(C-T)[29]. The mycobacterial resistance to FQ is caused by a mutation in the *gyrA* and *gyrB* gene which encodes DNA gyrase, the enzyme involved in DNA synthesis[30]. 42% to 85% of FQ-resistant *M. tuberculosis* strains have mutations in the *gyrA* gene, located in a 120 bp region called quinolone resistance determining region (QRDR), more often in codons 94, 90, 91, and 88[2,31,32]. High level resistance to the second-line injectable drugs KAN and AK is caused by mutations at codons 1400, 1401, 1322 or 1484 of *rrs* gene implicated in protein synthesis[32,33]. Mutations in codons 1401, 1402, 1473 and 1484 of the same genes associate resistance to CAP[19,29,33]. Also, the resistance to KAN is induced by mutations in codons 2, 10, 14, and 37 of *eis* gene which encodes an aminoglycoside-acetyltransferase that inactivates KAN by acetylation[33,34]. Molecular techniques use amplification of nucleic acid and detection by electrophoresis, hybridization or sequencing of alleles related to drug resistance, both in culture and in sputum[2]. Direct detection in sputum shortens the time needed to obtain results because cultivation is no longer necessary.

The first rapid molecular tests recommended by WHO for RIF and INH resistance detection were the rapid line probe assays (LPA): GenoType MTBDR assay (Hain Lifescience,

Germany) and INNO-LiPA Rif. TB kit (Innogenetics, Belgium) for TB patients with smear positive sputum samples[2,35]. These tests are based on DNA amplification of mycobacterial resistance determinants by polymerase chain reaction (PCR), followed by hybridization to strips containing specific probes for wild-type and mutated sequences of genes involved in drug resistance[2,6]. Both commercial kits can detect the most frequent mutations in core region of *rpoB* gene associated with RIF resistance and the GenoType MTBDR assay is also able to detect the INH resistance caused by mutations in *katG*315[15]. The results are obtained within 5 hours, with a cost of almost 20-22 $/sample[6,15,36]. INNO-LiPA Rif. TB has a high sensitivity (>95%) and a specificity of nearly 100% if the test is applied in culture isolates, but the test accuracy is variable for sputum[15]. GenoType MTBDR assay has high sensitivity for RIF resistance detection, but only 70-90% sensitivity for INH resistance, while the specificity is nearly 100% for both drugs[2]. GenoType MTBDR*plus* is an advanced version of the assay that is able to additionally detect the wild type of *rpoB* gene and also mutations in the promoter region of *inhA* gene involved in low-level INH resistance[2,6], increasing the test sensitivity for INH resistance detection with 10-20%[2]. This test can shorten by 25 days the time required to initiate the specific therapy for MDR-TB[6,37]. Although LPA are useful for rapid detection of drug resistance directly in sputum, these tests have a number of disadvantages: they cannot detect newly emerging mutations or differentiate between silent mutation (without phenotypic expression) and acquired resistance[15]. Also, these tests require special equipment, making them less accessible to countries with limited resources[2]. Yet, in 2016, WHO reiterated his recommendation for the use of two new rapid LPA for the detection of resistance to INH and RIF: MTBDR*plus* Version 2 (Hain LifeScience, Germany) and the Nipro NTM+MDRTB detection kit 2 (Nipro Corp., Japan)[1]. The Genotype® MTBDR*sl* version 1.0 was developed in 2009 and was designed to detect resistance to FQ, the second-line injectable drugs (AK, KAN, CAP) and ethambutol by identifying mutations in *gyrA*, *rrs* and *embB* genes, respectively. The test showed a good sensitivity for detecting resistance to FQ, AK and CAP (between 82% and 87%), but not for KAN (44%) and ethambutol (67%)[38]. The performances of test for FQ resistance detection are similar in culture (sensitivity: 83.1%, specificity: 97.7%) as in sputum samples (sensitivity: 85.1%, specificity: 98.2%)[39,40]. For AK, KAN and CAP resistance detection, the test performance is higher in sputum (sensitivity: 94.4%, specificity: 98.2%) than in culture (sensitivity: 76.9%, specificity: 99.5%)[39,40]. Due to the low sensitivity of ethambutol resistance detection, the Genotype® MTBDRsl version 2.0 (2015) no longer identified *embB* gene mutations. Also, in order to improve the detection of FQ resistance and KAN low level resistance, the test included the additional detection of *gyrB* and *eis* mutations respectively, beside that of *gyrA* and *rrs* mutations. Thus, the test sensitivity for identification of FQ resistance has increased, both for sputum (93%) and for culture (83.6%). The sensitivity of KAN resistance detection reached values of 96% in sputum and 95.5% in culture[41]. In 2016, WHO recommended the use of Genotype® MTBDRsl as initial test for XDR-TB diagnosis in confirmed RR/MDR-TB cases instead the phenotypic DST[1].

Another rapid molecular test used for MDR-TB diagnosis is Xpert® MTB/RIF assay (Cepheid, Sunnyvale USA) which can simultaneously identify *M. tuberculosis complex* and detect RIF resistance[42]. The target sequences are represented by 15-20 bp fragments of the wild-type core region in *rpoB* gene, that are amplified by real-time PCR and hybridized using different complementary fluorescent probes. The concordance target-probe hybridization determines a fluorescent signal, but in the presence of mutation, the probe no longer recognizes the target sequence and fluorescence is inhibited. Thus, the test can also point out new mutations[6,15]. In 2010, WHO recommended this test for diagnosis of MDR-TB and HIV-associated TB in adults, and in 2013, extended recommendation for TB in children and extrapulmonary TB cases[1]. The Xpert Mtb/RIF test showed high sensitivity and specificity compared with culture (86-100% and 95-100%, respectively) especially for the detection of RIF resistance directly on smear positive sputum[6,15,43]. This automated test avoids cross-contamination, it is easy to do and implement, it is fast (it generates results in 2 hours) and has a low cost (10$/cartridge)[1,6,15,44]. Because the test only detects the RIF resistance, it has the disadvantage that strains with monoresistance to RIF can wrongly be classified as MDR-TB, and strains with monoresistance to INH are not detected, causing an inadequate treatment[6,45,46,47]. Another disadvantage of the test is its inability to indicate mutations located outside the core region of *rpoB* gene[15].

Regarding the genetic detection of MDR/XDR-TB, the different types of DNA sequencing (i.e., whole-genome sequencing, pyrosequencing) are able to detect multiple mutations, but are expensive, difficult to apply in sputum and not used yet as a diagnosis tools[6,15,48].

Laboratory diagnosis of MDR-TB and XDR-TB in Romania

There were 650 estimated MDR-TB cases in Romania in 2014 (490-810 cases). DST for first-line anti-TB drugs was performed only in 77.2% of all pulmonary culture-confirmed TB cases. MDR-TB accounted for 6.4% (510 cases) of all pulmonary culture-confirmed TB cases, representing 2.1% of new pulmonary TB cases and 17.8% of previously treated TB cases[10]. DST to second-line anti-TB drugs was performed for 54.3% of MDR-TB cases (277 cases) and 58 cases (20.9%) were confirmed as XDR-TB[10]. TB diagnosis was conducted in 105 laboratories: 14 of level I, 48 of level II and 43 of level III (including two national TB reference laboratories). Only level III laboratories have ensured DST for RIF and INH through culture on solid-media and the national TB reference laboratories have applied the DST for first and second-line anti-TB drugs. Automated liquid culture systems (BACTEC MGIT; MB/BacT; Versa Trek) were available in 15 laboratories, but their use was limited by the discontinuous supply of consumables. LPA for RIF and INH resistance were applied in 4 laboratories (Bucharest, Cluj, Brașov and Constanța) and for second-line anti-TB drugs only in the national TB reference laboratories[49]. In order to increase the detection rate of DR-TB cases, the bacteriology laboratory network was reorganized. The activities of level III laboratories were supplemented with detection of RIF resistance by Xpert® MTB/RIF

assay. Also, 8 regional reference laboratories have been created, to perform first-line DST using automated liquid culture systems and LPA. The two national reference laboratories will also perform the role of regional laboratories. In addition, they will perform DST to second-line anti-TB drugs and will coordinate the entire network of laboratories[50]. To ensure universal access to rapid diagnostic methods for DR-TB, the National Strategy for Tuberculosis Control in Romania 2015-2020 plans to increase up to 9 the number of laboratories that use MGIT/LPA and recommends the use of GeneXpert assay in 42 laboratories for the identification of *M. tuberculosis* and RIF resistance for risk groups[51].

Conclusions

The emergence of DR-TB increases mortality and threatens the progress achieved in the management of TB. The diagnosis of DR-TB by classical phenotypic methods is time-consuming, resulting in delays in initiating effective treatment and promotes the spread of these strains. These disadvantages are adjusted by molecular methods able to identify the most common mutations that induce drug resistance, but these methods require trained personnel and special equipment. Universal access to rapid diagnostic methods for M/XDR-TB involves the use of molecular tests, but also implies a low cost to ensure accessibility in resource-limited countries. ■

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