LPA or GeneXpert in the diagnosis of multidrug-resistant tuberculosis

LPA sau GeneXpert în diagnosticul tuberculozei chimiorezistente

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Abstract

Facing a constant increase of multidrug-resistant tuberculosis (MDR-TB), there is large need for routine use of new diagnostic tests, based on molecular techniques that allow both a rapid diagnosis for TB complex and rapid identification of resistance mutations. The resistances are due to genetic factors: accumulation of changes within the genome structure, acquisition or loss of genes, spontaneous mutations in chromosomal genes, and changes that induce selection of resistant strains during a suboptimal treatment. The bacteriology laboratory plays a crucial role in the making of the diagnosis, monitoring and preventing TB transmission. World Health Organization offers consistent recommendations in favour of use of Xpert MTB/RIF, GeneXpert platform, as initial diagnostic test in adults and children suspected of TB, because it can simultaneously determine the presence of Mycobacterium tuberculosis and the Rifampicin resistance, which is a surrogate marker of MDR strains. The very high sensibility and specificity, also in the smear negative samples, as well as the short time needed for the results, make Xpert MTB/RIF a valuable tool in the fight against TB. Other recommended tests are: LPA, which identifies M. Tuberculosis complex, the Rifampicin and Isoniazid resistance; MTBDR plus or, for second line anti-TB drugs, the MTBDRsl.

Keywords: ***molecular methods, Rifampicin resistance, TB rapid diagnosis, LPA, Xpert MTB/RIF***

Rezumat

În contextul în care incidența cazurilor de tuberculoză multidrog rezistentă (MDR-TB) a crescut, se impune utilizarea de rutină a testelor de diagnostic noi, bazate pe tehnici moleculare care permit diagnosticul rapid atât pentru complexul TB, cât și pentru depistarea precoce a mutațiilor de rezistență. Rezistențele sunt datorate factorilor genetici: acumulările de schimbări în structura genomului, achiziția/pierderea de gene, mutațiile spontane în genele cromozomiale, modificări care produc selecția tulpinilor rezistente în timpul terapiei suboptimale. Laboratorul joacă un rol crucial în stabilirea diagnosticului, monitorizarea terapiei și prevenirea transmiterii tuberculozei. Organizatia Mondiala a Sănătății dă recomandări ferme pentru utilizarea Xpert MTB/RIF, platforma GeneXpert, ca test de diagnostic inițial la adulți și copii suspecți de TB deoarece determină simultan atât prezența M. tuberculosis (MTB), cât și rezistența la Rifampicină, marker surogat al tulpinilor MDR. Sensibilitatea și specificitatea foarte ridicate, chiar și în cazul sputelor cu microscopie negativă, cât și timpul foarte scurt în obținerea rezultatelor face din Xpert MTB/RIF un aliat valoros în lupta anti-TB. Alt test de diagnostic recomandat este LPA, care determină complexul MTB, rezistența la Rifampicină și rezistența la izoniazidă, MTBDRplus sau, pentru antituberculoase de linia a II-a, MTBDRsl.

Cuvinte-cheie: ***metode moleculare, rezistență RIF, diagnostic rapid TB, LPA, Xpert MTB/RIF***

The slogan of this year's World TB Day was "We Will END TB", while the vision of the strategy for global TB elimination is: "A world without tuberculosis, without death, disease and suffering due to tuberculosis". The aim of the strategy is: "The end of TB epidemic at global scale".

In 2014, the World Health Organization (WHO) reported over 9 million people worldwide having tuberculosis and 1.5 million people dying of the disease, making it a leader within the five deadliest infectious diseases. Of the total of TB cases, 5% are estimated to be multidrug-resistant TB (MDR-TB), while tuberculosis with extended resistance, XDR-TB, was reported in 105 countries in 2014, estimating that 9.7% of the total MDR-TB patients have XDR-TB[1].

In 2016, the global objective of "End TB" strategy is to put an end to the TB epidemic worldwide. In this respect, Dr. Margaret Chan, general director of WHO, declared: "Any person with TB should have access to the innovative instruments and services needed for rapid diagnosis, treatment and care. This is a matter of social justice, fundamental for our objective of universal cover of health. Taking into account the prevalence of drug-resistant tuberculosis, by ensuring complete and high quality medical care, we will benefit also of health security worldwide. I appeal for solidarity at global scale for intensifying the measures needed to ensure the success of this End TB Strategy"[2].

The global elimination of TB as a public health problem, defined as less than 1 TB case in 1 million inhabitants, is the long term vision of the End TB Strategy of WHO, while the timeline global objective is to "put an end to the TB epidemic worldwide", defined as decreasing the global incidence from > 1,000 in 1 million inhabitants in 2015 to < 100 in 1 million inhabitants before 2035[2]. "Early TB diagnosis, including the universal drug susceptibility testing, the use of rapid tests and systematic screening of contacts and risk groups are priorities of the strategy"[2].

The most important objectives of the strategy are: decreasing the TB incidence by 80% before 2030, and decreasing TB deaths by 90%[2].

MDR-TB is a formidable challenge for TB control due to the complex diagnosis and treatment challenges. The global annual burden of MDR-TB is estimated at about 490,000 cases (5% of total TB cases)[1].

Nevertheless, nowadays it is estimated that less than 5% of existing MDR-TB patients are diagnosed, as a consequence of serious limitations of laboratory capabilities.

The alarming increase of MDR-TB, as well as the emergence of XDR-TB and the rapid mortality of patients with HIV and MDR or XDR-TB co-infection, stressed upon the emergency for developing rapid screening methods[3].

In Romania in 2014 there were 12,498 new TB cases and 1,125 deaths. Mortality rate by TB was 5.7 in 100,000 inhabitants, higher than the European mean of 5.3%. There were 510 cases of MDR-TB, with 123 newly registered cases. There were 58 registered cases of XDR-TB[4,5].

As drug-resistant TB is becoming a global public health problem, the rapid diagnosis techniques become mandatory. In 2008, in "Policy Statement", WHO recommended the use of Line Probe Assay (LPA) technique for the rapid screening of patients at risk for MDR-TB[3].

Conventional methods for diagnosing TB and for testing the germs susceptibility to drugs are slow and laborious, involving successive processing steps, in order to obtain first the strain and then to test the susceptibility of the germs to anti-TB drugs. Meanwhile, the patients risk being treated inappropriately, and continuing to spread the disease and amplifying the resistance phenomenon[3].

LPA was the first test using amplification of nucleic acid (NAAT) approved by WHO. The test uses molecular detection of *M. tuberculosis*, in the same time detecting the resistance to Isoniazid (INH) and Rifampicin (RIF), the two major anti-TB drugs. The test is recommended for patients with positive sputum smear for acid-fast bacilli, having also a suspicion of MDR-TB. LPA tests were developed for the identification of mycobacteria, non-TB mycobacteria and screening of drug-resistance[6].

The basic principle of LPA is a multiplex amplification of DNA: several pairs of specific biotinated primers are included in the reaction. Primers are nucleotide sequences complementary to the ends of a DNA chain. The technique consists of three major steps: **1.** DNA extraction; **2.** DNA amplification; **3.** Reverse hybridization. The strips are marked with specific oligonucleotide probes, complementary to the target sequence of the DNA. After chemical denaturation, the monocatenar amplicon will bind to the specific complementary probe.

DNA extraction can be done directly in the smear positive sample, or indirectly from the strain isolated by culture. DNA targets are selectively amplified and marked with biotine. Next, a single monocatenar amplicon is applied on the test strip. Oligonucleotide specific probes are fixed on a nitrocellulose base, which subsequently is cut into test strips. Each amplicon binds specifically only to the complementary probe on the strip, while unbinded amplicons are removed in the next washing step. Bonded amplicons are detected by "attaching" a label (a bonded enzyme – streptavidin) to the biotinated amplicons, that induce a colour reaction. A series of dark bands mark the regions of the strip where the amplified DNA is bound. The result can be visually evaluated using a grading diagram, or using a reader. The strips also include the quality controls for amplification and hybridization. LPA tests are created to be used in reference and intermediary level laboratories. The technique used can be manual or semi-automatic for the DNA extraction, hybridization, washing and, finally, reading and evaluating the test results. A thermo cycler is needed for amplification of the extracted DNA samples.

Initially, difficulties due to frequent contamination of testing areas and subjective reading of strips were noted. Contamination can be reduced by improving the quality control procedures, by restricting the access in areas dedicated to molecular diagnosis, by strictly following the standard operational procedures and automatization of the hybridization process.

The main advantage of LPA is that, besides detecting mycobacteria, it can identify the species and can obtain information on the susceptibility to drugs of the strain by evidencing the genetic changes related to resistance. The results are usually available in 24 hours.

LPA can be used for a variety of biological samples. Smear positive samples as well as cultured strains offer better results due to more adequate DNA quantities.

HAIN Lifescience MTBDRplus version 2.0 is a modified version of the original test, approved by WHO in 2008. By increasing the efficiency of the polymerase chain reaction (PCR), the manufacturers claim to obtain a better sensibility also for smear negative samples, that later get culture positive. In a study, MTBDRplus, version 2.0 was proved to have a similar performance to Xpert MTB/RIF[6].

In March 2012, a group of WHO experts revised the performance of the first LPA method for genotyping of alleles associated to second line drugs resistance (fluoroquinolones, cyclic peptides and Etambutole), namely Hain Lifescience GenoType®. The test is designed as a cheap and rapid instrument for identification of XDR-TB among MDR-TB specimens in a reference laboratory, in order to replace the phenotypic susceptibility tests. After analysing the data collected, the group of experts decided that the performance of GenoType®MTBDRsl test was not good enough to completely replace the phenotypic methods[6].

Nevertheless, the experts noted that, taking into account the high specificity for detecting the resistance to fluoroquinolones (FLQ) and second line injectable drugs, the results of MTBDRsl test could be used as a rapid test to guide supplemental measures for infection control, while waiting for the results of the phenotypic susceptibility tests. A recent study performed in Congo noticed that a common mutation in the strains susceptible to FLQ could be interpreted as a marker for FLQ resistance. There is need for improving the design of this test, to have better information in case these alleles are found[7].

LPA tests show several benefits: they are sensitive, can be used for a variety of purposes: identification of mycobacteria, diagnosis of strains in TB complex, species diagnosis for non-TB mycobacteria, genotyping of common alleles for drug resistance (especially for RIF) in a short time (24 hours). The tests offer a great amount of information in a single test with relatively low costs. Generic equipment can be used, like "amplifiers", and testing can also be performed on semi-automatic systems[6].

LPAs also have some drawbacks. Tests can only be performed in reference and regional laboratories because of the need for trained personnel, special conditions for storing the supplies and special equipment. To reduce the risk of contamination, dedicated spaces are needed[8]. For smear negative/culture positive samples it is recommended to use only Hain Lifescience GenoType® version 2.0. Manual reading and evaluation of results needs increased attention in order to avoid errors. Like other molecular susceptibility tests, resistance mutations to several drugs cannot be identified (e.g.: for pyrazinamide). On the other hand, silent mutations in susceptible strains can be incorrectly interpreted as drug resistance, leading to prescription of expensive and un-needed treatments. The new technologies are not used extensively, so the evidence regarding their applicability is limited[6].

For second line drugs, a new version pf LPA was developed (LPAsl), including probes for the detection of mutations in genes *gyrA* and *gyrB* and *eis* promoter, associated to resistance to fluoroquinolones. The presence of mutations in these regions does not presume cross reaction to all the drugs in the same class, and the specific mutations can be associated to different levels of resistance (different minimal inhibitory concentrations) for each drug of the group. The level of cross reaction is not fully understood. More information is needed to better understand the correlation between the genotypic resistance, marked by the presence of resistance mutations for quinolones, and the phenotypic resistance. This information needs to be correlated with the patient's clinical data.

The test can be performed directly, using a processed sputum sample (sediment obtained after centrifugation), or indirectly, using the DNA extracted and amplified, originating from a *M. tuberculosis* culture. The direct testing consists of: **1.** Decontamination; **2.** Extraction and amplification of DNA; **3.** Reverse hybridization for detection of amplification products, and **4.** Visualising by means of a colour reaction using a conjugate (streptavidin).

Each band corresponds to a wild type probe or to a resistance mutation. They can be used to determine the signature of drug susceptibility of the sample. The test can be performed in a single day.

It is debatable if the clinician should use the result of LPAsl test to guide the clinical use of FLQ or other second line drugs in patients with Rifampicin resistant or MDR-TB, instead of the phenotypic susceptibility test, which is accepted as reference standard. The results are confident only when an appropriate drug concentration is used to determine the threshold that makes the difference between probably susceptible and probably resistant strains[7].

Under these circumstances, WHO recommends TB patients with RIF or MD resistance using LPAsl as an initial diagnostic test instead of phenotypic antibiogram, for detecting the FLQ resistance[7]. For injectable second line drugs, there is a conditional recommendation by WHO to use LPAsl as an initial test instead of a phenotypic antibiogram, due to moderate or even low evidence of the accuracy of the test[7].

The development of Xpert MTB/RIF test for GeneXpert diagnostic platform was finalized in 2009 and is considered a major progress in the fight against TB. For the first time, a molecular test is simple and robust enough to be used outside the specific conditions of a dedicated laboratory.

Xpert MTB/RIF detects *M. tuberculosis* (MTB), as well as the mutations giving RIF resistance. The method uses three specific primers and five unique molecular probes, to ensure a high level of specificity. The test offers results directly from sputum in less than 2 hours. GeneXpert remains the single technology able to independently test DNA in a cartouche in a completely automated platform, able to detect accurately TB and Rifampicin resistance in less than 2 hours[9].

Here are the main recommendations of WHO, issued in 2013, regarding the use of Xpert MTB/RIF for the diagnosis of TB and RIF resistance in adults and children:

- Xpert MTB/RIF should be used as an initial diagnosis test, instead of conventional microscopy, culture and phenotypic antibiogram, in adults suspected of having MDR-TB or TB associated to HIV infection (strong recommendation, high quality evidence is available).
- Xpert MTB/RIF should be used as an initial test, before conventional microscopy, culture and phenotypic antibiogram, in children suspected of having MDR-TB or TB associated to HIV infection (strong recommendation, low quality evidence available).
- Xpert MTB/RIF can be used as an initial diagnostic test, before conventional microscopy, culture and phenotypic antibiogram, in all adults and children suspected to have TB (conditional recommendation, admits the need for high resources, low evidence).
- Xpert MTB/RIF can be used following microscopy in adults suspected to have TB, not exposed to MDR-TB or HIV risk, especially when the subsequent tests show negative microscopy (conditional recommendation, high quality evidence).

Conventional microscopy and culture remain essential for treatment follow-up and for performing the phenotypic antibiogram, in order to define the susceptibility profile for anti-TB drugs besides Rifampicin (including Isoniazid and second line drugs)[9].

The results of analytic studies showed that Xpert MTB/RIF has an analytic sensitivity for 5 copies of purified DNA genome and 131 cfu/ml *M. tuberculosis* in sputum. Molecular probes that target *rpoB* gene cover all the known mutations in more than 99.5% of all strains resistant to Rifampicin. There is no cross reactivity with

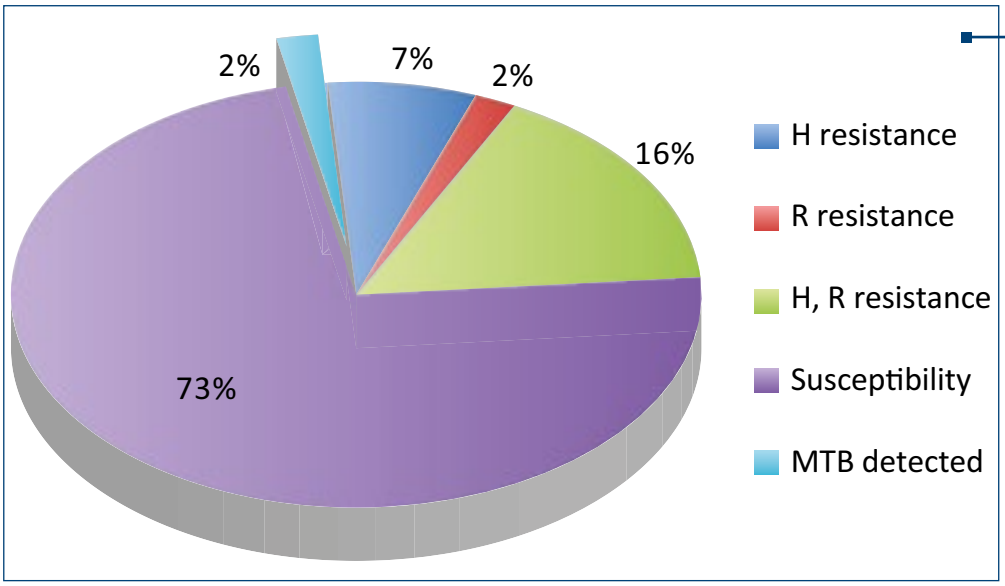

Figure 1. *Distribution of resistance detected by first line LPA (773 strains)*

non-TB and TB mycobacteria, and the Rifampicin resistance was correctly detected in the presence of DNA from non-TB strains or from a mixture of susceptible and resistant strains. When the reactive for sputum processing is added in a ratio of 2:1 to the sputum, it destroys more then 6 $\log_{10}$ cfu/ml of *M. tuberculosis* in a 15 minutes' exposure, so more than 97% of the smear positive samples become negative when using Löwenstein-Jensen cultivation. During inoculation and testing, no infectious aerosols are generated [9].

When used as initial diagnostic test, replacing the microscopic testing, Xpert MTB/RIF reaches a cumulative sensitivity of 88% (95% credibility interval [Cri] 84-92%) and a cumulative specificity of 99% (95% [Cri], 98-99%), as proven in 22 studies including 9008 participants. When it is used as a supplemental test to follow a negative microscopic test, Xpert MTB/RIF cumulates a sensitivity of 68% (95% [Cri], 61-74%) and a cumulative specificity of 99% (95%) [9].

Xpert MTB/RIF offers several benefits:

- It simultaneously detects the presence of *M. tuberculosis* and the Rifampicin resistance in less then 2 hours;
- The sensibility for TB detection is similar to the liquid cultivation (sensibility of 88% as compared to the liquid culture); the specificity is also high (99%);
- For smear negative/culture positive TB, the sensibility of Xpert MTB/RIF is 68%. The superior performance over microscopy of Xpert MTB/RIF in detecting TB makes it a most valuable tool for TB diagnosis in patients with HIV co-infection. For the detection of rifampicin resistance, the test has a sensibility of 95% and a specificity of 98% when compared to phenotypic tests.
- The biosafety measures required by Xpert MTB/RIF are similar to those needed for a microscopy smear, and the production of infectious aerosol is minimal. This makes this technique suitable also for low grade laboratories[10].

Nevertheless, Xpert MTB/RIF has also some drawbacks:

- It needs a continuous and stable electric power supply. In case of blackouts supplemental batteries or UPS systems are needed to provide the energy supply for 2 hours.
- The ambient temperature cannot exceed 30°C, while the cartouches have to be stored at less than 28°C.
- The valability term of the cartouches needs to be monitored for preventing expiration before use. Attentive planning and management of re-supply are crucial.
- Security measures must be applied for preventing theft of the computer[10].
- The modules have to be calibrated annually; if they don't pass the calibration test performed with a specific calibration cartouche, they must be changed by importing extra modules, with significant extra costs.

The limits of using Xpert MTB/RIF are:

- The use of this technique doesn't exclude the need for conventional microscopy, culture and antibiogram, which are useful for treatment monitoring and detecting resistance to other drugs, besides rifampicin.
- In patients with no risk of drug resistance with an initial positive Xpert MTB/RIF test for rifampicin resistance, a second Xpert MTB/RIF test has to be performed in order to control pre-analytical and post-analytical errors and to increase diagnostic confidence.
- Increasing number of tests showed that frequent false-positive results can be related to identification of genuine RIF resistant strains, but with no phenotypic detection of the resistance, in the situation when phenotypic antibiogram is considered as reference. These strains display clinically significant mutations in the region for RIF resistance, which may actually lead to failure of the first line treatment.

When discrepancies between Xpert MTB/RIF, phenotypic antibiogram and LPA occur, the strain should be referred to a reference laboratory for sequencing. While waiting for the results, the clinician should decide to treat using a regimen for MDR-TB[10].

Here are some results from our experience.

In 2015 we performed 872 first line LPA tests, detecting MTB in 773 tests (88.64%). Among the 773 strains, 54 displayed single resistance to isoniazid (H), 14 single resistance to rifampicin (R), and 126 had HR resistance (figure 1).

There were 561 susceptible strains, while in 18 tests only TB complex was detected (invalid tests). For Xpert MTB/RIF, among 1580 tests performed, 314 (19.87%) detected MTB and 1266 did not. Among the 314 strains tested, 30 displayed Rifampicin resistance.

Conclusions

Molecular tests are no replacement for phenotypic culture or antibiogram. Culture is still needed for smear negative samples, and phenotypic antibiogram is needed to confirm XDR-TB.

Nevertheless, the use of molecular tests for the screening algorithm of MDR-TB can significantly reduce the expenditure with classic phenotypic tests for culture and antibiogram[11]. The patient can highly benefit from a fast diagnosis if tests are rapidly interpreted and reported to the clinician.

Multidrug-resistant tuberculosis is a serious threat, and understanding the pathways of drug resistance is crucial for reversing the ascending trend of this disease. If standard nationwide approved therapeutic regimens are applied, the cure rate is high, with only few relapses and drug resistances emerging[11]. These regimens are efficient against drug resistance, as combined chemotherapy makes less probable the occurrence of a mutant strain resistant to all components of the therapy. Patients with susceptible tuberculosis that are treated with inadequate regimes have the risk for selecting resistant mutants, because bacteria may be exposed to monotherapy. Initially, resistance to a single drug appears, followed by resistance to several drugs, ruining the protection offered by combined chemotherapy.

It is important to note that resistant strains are fully virulent, and epidemic occurrence of tuberculosis with multiple drug resistances needs to be prevented, by prompt diagnosis and efficient treatment, to stop the newly selected strains to be spread into the community[11].

There still is the unanswered question of what to do when there are discrepancies between molecular and phenotypic tests, the latter being the gold standard.

The level of discrepancy depends on the drug and the genomic region tested. Despite the fact that the phenotypic antibiogram result is not always concordant to the clinical outcome after treatment, still the phenotypic methods for evaluation of susceptibility remain the golden standard[11].

What is the consequence of identifying a resistance in a second line molecular test in a patient in whom the diagnostic is not confirmed and the result of phenotypic antibiogram is still awaited? The clinician should guide the treatment based on the molecular tests for rifampicin, isoniazid, etambutole, fluoroquinolones and injectables and start a regimen with second line drugs, while waiting for the results of the phenotypic test[12].

Molecular tests do not replace the phenotypic tests, which remain the "golden standard", but offer valuable information regarding the diagnosis and resistances, and guide the early start of targeted treatment, limiting the spread of resistant strains. ■

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ERATUM

For two articles published in Pneumologia in 2015, the affiliation of the first author (Daniel Traila) was printed incorrectly. The correct affiliation of the first author is as follows:

Pneumologia 2015, 64(1):8-13 – **Short telomeres in pulmonary fibrosis: from genetics to clinical significance**

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Pneumologia 2015, 64(2):37-40 – **Interstitial lung disease as first clinical manifestation within the antisynthetase syndrome – dermatomyositis**

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