REVIEW ARTICLE

Recent advances in molecular methods for early diagnosis of tuberculosis and drug-resistant tuberculosis

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Tuberculosis (TB) remains the main infectious cause of deaths in the world. Due to the slow metabolism of the causative agent, *Mycobacterium tuberculosis*, the isolation, identification and drug susceptibility testing requires several weeks. New techniques have improved specificity, turnaround time and cost effectiveness. Although these methods yield results within hours from sample collection, the clinical significance of each positive result requires rigorous evaluation in most cases. Herein the advantages and disadvantages of the most promising molecular techniques for detection of TB and drug resistance are discussed.

Key words: tuberculosis, diagnosis, mycobacteriophages, PCR, drug resistance, E-MTD.

Avances recientes en métodos moleculares para el diagnóstico precoz y tuberculosis resistente al tratamiento

La tuberculosis sigue siendo la principal causa de mortalidad por un agente infeccioso a escala mundial. Debido al metabolismo lento de su agente etiológico, *Mycobacterium tuberculosis*, el aislamiento, la identificación y las pruebas de susceptibilidad tardan varias semanas. Nuevas técnicas moleculares desarrolladas ofrecen mejorías en la especificidad, el tiempo para la obtención de resultados y su costo-efectividad. Estas pruebas producen resultados en pocas horas a partir de la toma de muestra, pero su relevancia clínica requiere aún ser evaluada rigurosamente en la mayoría de los casos. En esta revisión se discuten las ventajas y las desventajas de las pruebas moleculares más promisorias desarrolladas para el diagnóstico de la tuberculosis y la tuberculosis resistente a medicamentos.

Palabras clave: tuberculosis, diagnóstico, resistencia a fármacos, micobacteriófagos, PCR, E-MTD.

Tuberculosis (TB) remains the world's leading infectious cause of death in adults. The death toll is worsened by the emergence of drug resistant *Mycobacterium tuberculosis*, the causative agent of TB (1). The first report on drug resistance TB was described shortly after the introduction of anti-TB chemotherapy (2). Only a year later the phenomenon of antibiotic resistance was clearly illustrated when the British Medical Research Council showed that mortality in TB cases was similar in patients treated or untreated with streptomycin. Such observation led to the conclusion that TB should be treated with a regimen composed of more than one antibiotic (4). The emergence of multi-drug resistant (MDR) strains was observed during the subsequent decades with little attention by health authorities. During the 1990s major MDR-TB outbreaks were reported in the USA, Russia, and other countries. In New York City in 1991, 19.0% of all prevalent clinical isolates were MDR [by convention, MDR isolates are resistant at least to isoniazid (INH) and rifampicin (RIF)] (5-7). Since then, drug resistance (DR) TB has been reported in almost all geographical settings, underscoring the importance of expanding and strengthening TB control efforts worldwide (1,5,8,9).

It is clear that efficient diagnosis of TB and DR are important factors to improve the TB control program. Early diagnosis can lead to smaller

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number of contacts per case and lower MDR rates, for which the molecular methods used either for detection of *M. tuberculosis* or diagnosis of DR have an advantage of yielding results in only hours, while conventional microbiological approaches require days to weeks. Some techniques have simple setup and analysis permitting their applications in clinical settings. In this review some of the most important or promising molecular techniques will be discussed.

Detection of *Mycobacterium tuberculosis*

Molecular techniques determine the presence of *M. tuberculosis* in clinical specimens by detecting specific nucleic acid sequences after being amplified. The Food and Drug Administration (FDA) has approved only two molecular techniques for direct detection of *M. tuberculosis* from clinical specimens, the Amplicor *M. tuberculosis* test (Amplicor; Roche Diagnostic Systems, Inc., Branchburg, NJ) and the Enhanced *M. tuberculosis* Direct Test (E-MTD; Gen- Probe, San Diego, CA).

Amplicor test

This technique detects the presence of the mycobacterial 16S ribosomal RNA (rRNA) gene by PCR amplification followed by an ELISA reaction. The clinical specimen is processed with the standard N-acetyl-L-cysteine-NaOH decontamination method, followed by amplification of the mycobacterial 16S rRNA gene. The presence of the biotin-labeled amplicon is done by an ELISA reaction, for which the PCR product is denatured and added to a microtiter plate in which there is an immobilized DNA probe specific to the target. Presence of the target-probe hybrid is revealed by addition of an avidin-horseradish peroxidase conjugate antibody, and revealed by addition of peroxide and 3,3',5,5'-tetramethyl-benzidine in dimethylformamide to form a color complex. The results are measured with a photometer. False-positive results produced by carryover contamination are prevented by the incorporation of dUTP coupled with uracil-N-glycosylase restriction. The complete process takes about 6.5 hours, and an automated version of the test called Cobas Amplicor is available. Several studies have evaluated the Amplicor test efficiency in clinical settings. It performed well

among acid-fast bacilli (AFB) smear-positive patients with a specificity of 99.0% and sensitivity between 80.0-92.0%, while it presents lower sensitivity values (40.0-73.0%) among smear negative patients (10-14). Moreover, FDA approved Amplicor only for AFB smear-positive respiratory specimens. The studies agree that the specificity of the culture is similar to Amplicor test in detecting *M. tuberculosis* in respiratory samples when using bacterial, histopathological, and clinical data as gold standards, but the sensitivity is reported either as similar to culture (~100.0%) (15,16) or lower (42-73%) (17).

E-MTD

The assay is based on the transcription mediated amplification system (TMA) developed by Kwoh et al. (18), which is described in figure 1. The mycobacterial rRNA from the target cells is released by sonication and amplified a billion fold by TMA. The reactions take place at a constant temperature, replacing the thermal cycles of a PCR. The RNA amplicon produced is detected in solution with an acridinium ester-labeled DNA probe, using a luminometer. The assay takes 3.5 hours to yield results after the clinical specimen is processed with the standard N-acetyl-L-cysteine-NaOH decontamination method.

E-MTD has FDA approval only for respiratory specimens, either AFB-positive or -negative. A comparison between E-MTD and Amplicor showed similar performance in detecting *M. tuberculosis*. However, Amplicor can be fully automated and has internal controls to detect PCR inhibitions, while E-MTD does not (19). The equipment availability in the reference laboratory might play a role in choosing either technique: Amplicor requires a thermocycler and a photometer (or the Cobas Amplicor apparatus) while E-MTD requires a heat block and a photometer.

The Centers for Disease Control and Prevention (CDC) designed general guidelines for the clinical applications of nucleic acid amplification assays for TB diagnosis (20). They recommend collecting sputum specimens on 3 different days for AFB smear and culture. The nucleic acid detection tests should be performed on the first sample, the first smear positive specimen, and additional

specimens if needed. According to the results obtained, there are 4 different scenarios: 1) if smear and amplification are positive, the patient is presumed to have TB; 2) in cases where smear is positive and nucleic acid amplification tests are negative, a test for amplification reactions inhibitors must be performed; in case inhibitors are detected, these assays are omitted; when there are no inhibitors and the smear is positive, the patient is presumably infected with non-tuberculous mycobacteria; 3) if the smear is AFB-negative with positive amplification, the second specimen is evaluated, and if similar results are observed the patient is presumed to have TB; 4) in cases where all sputum specimens are both smear and amplification negative, the patient can be presumed to be non-infectious, but in this case the possibility of active TB cannot be ruled out and the physician must evaluate the possibility of TB therapy (20,21).

Clinical evaluation of extrapulmonary TB is often uncertain and nucleic acid amplification techniques offer a new alternative to clinicians for accurate diagnosis of TB. Several studies report similar performance of Amplicor and E-MTD tests using extrapulmonary samples when using culture as gold standard (22,23) but there are reports in which performance is questioned (14). It is clear that further clinical evaluation is required before FDA approves these techniques for non-respiratory specimens, and caution must be practiced by physicians in these cases.

There are numerous in-house PCR-based techniques developed for the detection of TB in diverse clinical specimens with promising results (24-29). Such assays offer a TB diagnostic alternative in children with high agreement with standard techniques (30-32). On the other hand, some PCR assays can yield false negative results (33-35), underscoring the importance of further evaluation and validation of each assay.

Identification of antibiotic resistance-associated mutations

M. tuberculosis acquires drug resistance by antibiotic selection of mutations that occur randomly at chromosomal loci. No plasmids or transposable elements are involved in this process. Nucleotide changes (point mutations, small deletions or insertions) confer resistance to single drugs, and the stepwise accumulation of these mutations leads to MDR-TB. Drug-resistant strains emerge when chemotherapy is intermittent or otherwise inadequate, underscoring the importance of an early detection of DR. The increase in drug resistant TB and the limited number of therapeutic agents is renewing the effort in the study of the molecular mechanisms of resistance. Before discussing the methods designed to detect these mutations, a brief description of the molecular mechanisms of DR in *M. tuberculosis* will be made [for reviews, see references (36-38)].

RIF is a semisynthetic derivative of rifamycin that is used as a first-line TB drug. RIF binds to the β subunit of RNA polymerase, encoded by the *rpoB* gene, and inhibits transcription initiation. About 96% of RIF-resistant (RIF^R) isolates have point mutations in an 81-bp region of this gene, the *rpoB* core region, and these mutations are absent in susceptible isolates, making it an ideal target for development of molecular drug susceptibility testing methods (39,40). In contrast to RIF, the genetic basis of resistance to other TB drugs is more complex. INH is a synthetic, bactericidal agent that is used only for treatment of TB because basically all other bacteria are inherently resistant to INH. Alterations in a relatively large number of genes have been associated with INH resistance, but mutations in two genes, *katG* and *inhA,* are found in 75-85% of INH-resistant *M. tuberculosis* isolates. Streptomycin is an aminoglycoside antibiotic that inhibits protein synthesis. Approximately 65-75% of streptomycin-resistant *M. tuberculosis* isolates have mutations in the 16S rRNA gene or the *rpsL* gene, which codes for the ribosomal protein S12. More than 70% of the pyrazinamide-resistant *M. tuberculosis* isolates have mutations in the *pncA* gene, which encodes for pyrazinamidase, an enzyme that converts pyrazinamide to its active form. Ethambutol inhibits the incorporation of essential mycolic acids into the mycobacterial cell wall. Mutations in the *embB* gene are associated with ethambutol resistance in ~70% of resistant isolates (36-38).

The presence or absence of mutations in DR-

associated genes does not necessarily indicate susceptibility or resistance to the corresponding antibiotic. Reports of resistant isolates without mutations in DR-associated genes and reports of susceptible isolates with mutations in DR-associated genes indicate a partial understanding of resistance mechanisms (38,41). Several mutations have been stated as 'real' resistance-mutations (*i.e.* position 315 in *katG*) (36-38,42), while some have been stated as 'non-resistant mutations' [*i.e.* position 463 in *katG* (38, 43), and positions 269 and 312 of *kasA* (41,43,44)]. In contrast, the majority of mutations detected come from studies using samples with low statistical significance and their DR-association is based only on the resistant phenotype of the isolate. This underscores the importance of determining the role of each 'DR-associated' mutation reported. Cases where 'real' mutations associated with resistance are detected are clinically relevant, however their absence does not necessarily mean that the organism is susceptible to the drug in question. For this reason the currently available molecular methods may aid in rapid detection of such mutations, but the results must always be confirmed by phenotypic methods. Some of the most important techniques for detection of DR-associated mutations are described below.

DNA sequencing

Sequencing is the most accurate and reliable method for mutation detection, and it is used as the gold standard technique. It allows detecting both previously recognized and unrecognized mutations. Except for RIF, DNA sequencing is unlikely to be used in routine detection of DR mutations because it requires several sequencing reactions per isolate becoming labor-intensive and costly.

The Line Probe assay (LiPA)

LiPA (Inno-Genetics N.V., Zwijndrecht, Belgium) is used to identify *M. tuberculosis* species and the presence of mutations in the *rpoB* core region. This region is amplified and biotin-labeled by PCR. This amplicor is detected after hybridization with a strip in which five probes for wild-type *rpoB* sequences, four probes for specific *rpoB* mutations, a conjugate control, and *M.*

tuberculosis control probes were immobilized. Bound amplicons are then detected with a color reaction. Banding pattern interpretation allows *M. tuberculosis* complex identification and detection of *rpoB* mutations. The assay is designed to specifically detect 4 of the most common *rpoB* mutations [~75% of RIF[R] clinical isolates carry 1 of the 4 mutations (38)] as well as different mutations in the core region. The test can be performed on cultures or directly from clinical specimens in less than 48 hours. Overall concordance of the LiPA test with phenotypic susceptibility testing and direct sequencing, when performed from cultures, varies from 92.2% to
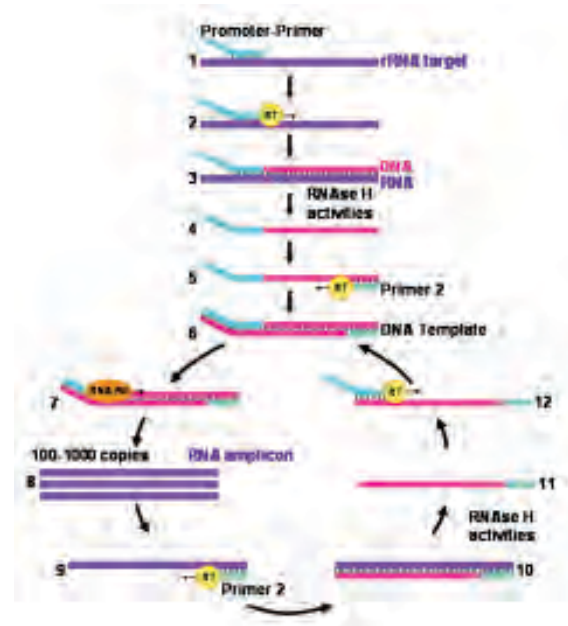

Figure 1. Amplification of the rRNA target from *M. tuberculosis* by TMA. 1: Promoter-primer binds to rRNA target. 2: Reverse transcriptase (RT) creates DNA copy of rRNA target. 3: RNA:DNA duplex. 4: RNAse H activities of RT degrades the rRNA. 5: Primer 2 binds to the DNA and RT creates a new DNA copy. 6: Double-stranded DNA template with a promoter sequence. 7: RNA polymerase (RNA Pol) initiates transcription of RNA from DNA template. 8: 100-1000 copies of RNA amplicon are produced. 9: Primer 2 binds to each RNA amplicon and RT creates a DNA copy. 10: RNA:DNA duplex. 11: RNAse H activities of RT degrades the rRNA. 12: Promoter-primer binds to the newly synthesized DNA. RT creates a double-stranded DNA and the autocatalytic cycle repeats resulting in a billion-fold am-plification. Detection of the RNA amplicon is performed in an independent reaction. Reproduced with permission of Dr. Craig S. Hill, Gen-Probe Inc., CA.

99.0% (46-48), making the LiPA test a useful method for rapid detection of RIF[R]. In cases where a mixed population of wild type/mutant is present, LiPA assay is able to detect the presence of the mutants only when mutants are at least 20% of the total population (49).

DNA microarrays

DNA microarrays are based on the principle of hybridization. They allow analysis of large amounts of DNA sequences with a single hybridization step. PCR amplicons labeled with fluorophore moieties are generated from the sample to be hybridized to a large collection of probes bound to a solid surface. The bound amplicons emit a fluorescent signal that is scanned with an epifluorescent microscope. Probes are designed to hybridize to fully complementary amplicons. Wild type and mutant probes are included in the array to determine the presence of specific mutations. Microarrays have been used mainly for species identification and for detection of mutations associated with RIF[R], with excellent concordance with sequencing results (50,51). New arrays are being developed covering several mutations in *katG, inhA, rpoB, rpsL* and *gyrA* proving that this approach has a strong potential for the screening of DR-associated mutations in *M. tuberculosis* (50). The widespread application of microarrays is limited because they are still under a research and development stage, require expertise and sophisticated equipment and are costly.

Molecular beacons

Molecular beacons are hairpin-shaped probes able to detect the presence of specific nucleic acids (figure 2). They are designed in such a way that the loop portion of the molecule is a probe sequence complementary to a target nucleic acid molecule. The stem is formed by the annealing of complementary arm sequences on the ends of the probe sequence. A quenching moiety is attached to the end of one arm and a fluorescent moiety is attached to the end of the other arm. The stem keeps these two moieties in close proximity to each other, causing the fluorescence of the fluorophore to be quenched by energy transfer. When the probe encounters a target molecule, it forms a hybrid that is longer and more
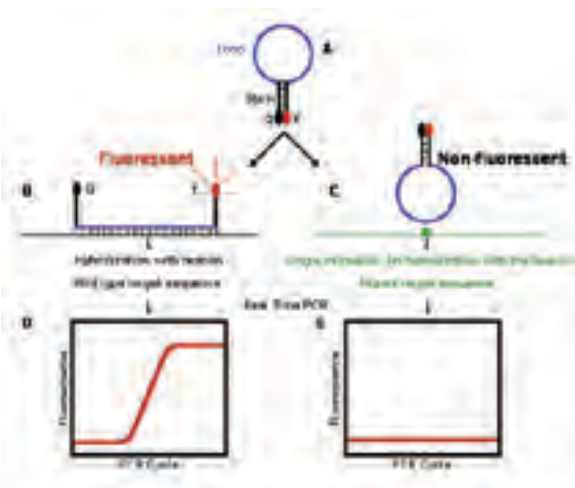

Figure 2. Molecular beacon assay. A molecular beacon has a loop portion complementary to a target nucleic acid molecule and a stem composed of 2 complementary tails, one with a quenching moiety (Q) and the other with a fluorescent moiety (F). A. When closed, fluorophore fluorescence is quenched by the quencher. B. When the probe encounters a target molecule, the beacon opens and hybridizes to it allowing fluorescence to be detected. C. In the event that the target has a mismatch (shown by a green box), the stability of the target-beacon is diminished and beacon remains closed in a non-fluorescent state. In case the PCR amplicon is complementary to the beacon, its amplification can be monitored by increase in fluorescence by real time PCR (D), otherwise no signal increase is observed (E).

stable than the stem hybrid and its rigidity and length preclude the simultaneous existence of the stem hybrid. Thus, the molecular beacon undergoes a spontaneous conformational reorganization that forces the stem apart, and causes the fluorophore and the quencher to move away from each other, leading to the restoration of fluorescence that can be detected. This is monitored in real-time, where the fluorescence increases every cycle in proportion to the amplification of the hybridizing target, which is not detected in cases when the target is not complementary to the beacon (52). Beacons are highly sensitive and specific: a single mismatch in the target sequence diminishes the beacon-target hybrid stability, allowing the detection of point mutations. Beacon assays are performed in sealed wells preventing amplicon contamination, they are easily implemented, automated and can be used in high throughput analysis. In the case

of RIF, a set of 5 beacons has been designed to cover the *rpoB* core region in a single reaction, with excellent results (53). The assay is sensitive enough to detect 2 bacilli, offers results in 3 hours from sputum collection, and identifies the *M. tuberculosis* species proving its strong potential in clinical settings. A different set of molecular beacons has been designed to screen for mutations in the regions with higher frequency of mutations associated with DR for INH [*katG* (position 315), the promoter region of *inhA*, the *oxyR-ahpC* intergenic region and positions 66, 269, 312 and 413 of *kasA*] (54). Results indicate that the assay is highly specific, but further research is required to identify the full set of mutations responsible for INH[R].

Single-strand conformation polymorphism (SSCP)

This technique determines the presence of mutations in specific DNA regions by their migration patterns in polyacrylamide gels. The target region of a gene is amplified by PCR and the product is denatured into two single-stranded molecules and subjected to nondenaturing polyacrylamide gel electrophoresis. Under non-denaturing conditions, the single-stranded DNA (ssDNA) molecule has a secondary structure determined by the nucleotide sequence, buffer conditions, and temperature. Mutant ssDNAs migrate to different positions than the wild type control. SSCP has been used to detect point mutations in *rpoB*, with promising results (40,55). However, there are reports of the presence of silent mutations (mutations that do not change the amino-acid sequence) in this region not resulting in RIF[R] leading to false positives by SSCP (56). This technique has risk of amplicon contamination due to the extensive post-PCR manipulation required. This technique is not practical for other antibiotics because they require screening larger DNA regions and more than one region per antibiotic.

FRET probes

Fluorescence resonance energy transfer (FRET) probes, also known as light cycler probes, are highly specific probes able to detect the presence of mutations in real time PCR reactions (57,58).

The detection system of light cycler probes is based on FRET with two different specific oligonucleotides (figure 3). Hybridization probe 1 is labeled with fluorescein, and hybridization probe 2 is labeled with the fluorophore Light Cycler Red 640. Both probes can hybridize in a head-to-tail arrangement, bringing the two fluorescent dyes into close proximity. A transfer of energy between the two probes results in emission of red fluorescent light. The level of fluorescence is proportional to the amount of DNA generated during the PCR process. In case the template is mutant, the probe-target stability will decrease and the probe will remain in solution hindering FRET, resulting in a flat signal. FRET probe assays for the rapid detection of INH[R] and RIF[R] were developed and tested with clinical isolates. The technique yields concordant results with sequencing data in less than 2 hours (57,58). This technique has the advantage that the region covered is larger than the region screened by molecular beacons, but because it entails the use of two fluorophore-labeled probes, the cost is doubled.

Other PCR-based techniques

Numerous in-house PCR approaches have been developed to detect the presence of specific point
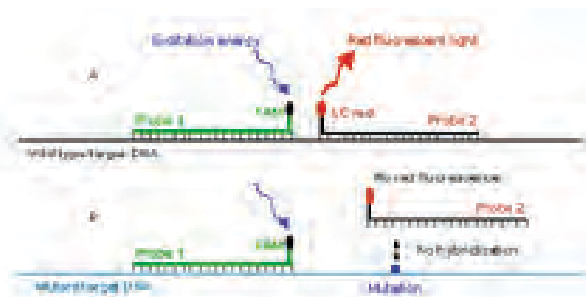

Figure 3. FRET probe assay. A. During the annealing step of a PCR reaction, target sequence hybridizes to probes 1 and 2 as shown in the figure. Probe 1 is labeled with fluorescein (FAM) and probe 2 with Light Cycler Red 640 fluorophore (LC red). Upon excitation of fluorescein by a light beam, the energy released is transferred to LC red by FRET and red fluorescent light is emitted. Red fluorescence increases proportionally to the amplification of the target DNA. B. Mutation presence (blue square) hinders probe hybridization, and no red fluorescence increase will be observed (similar effect will be observed when mutation is located in the region covered by probe 1).
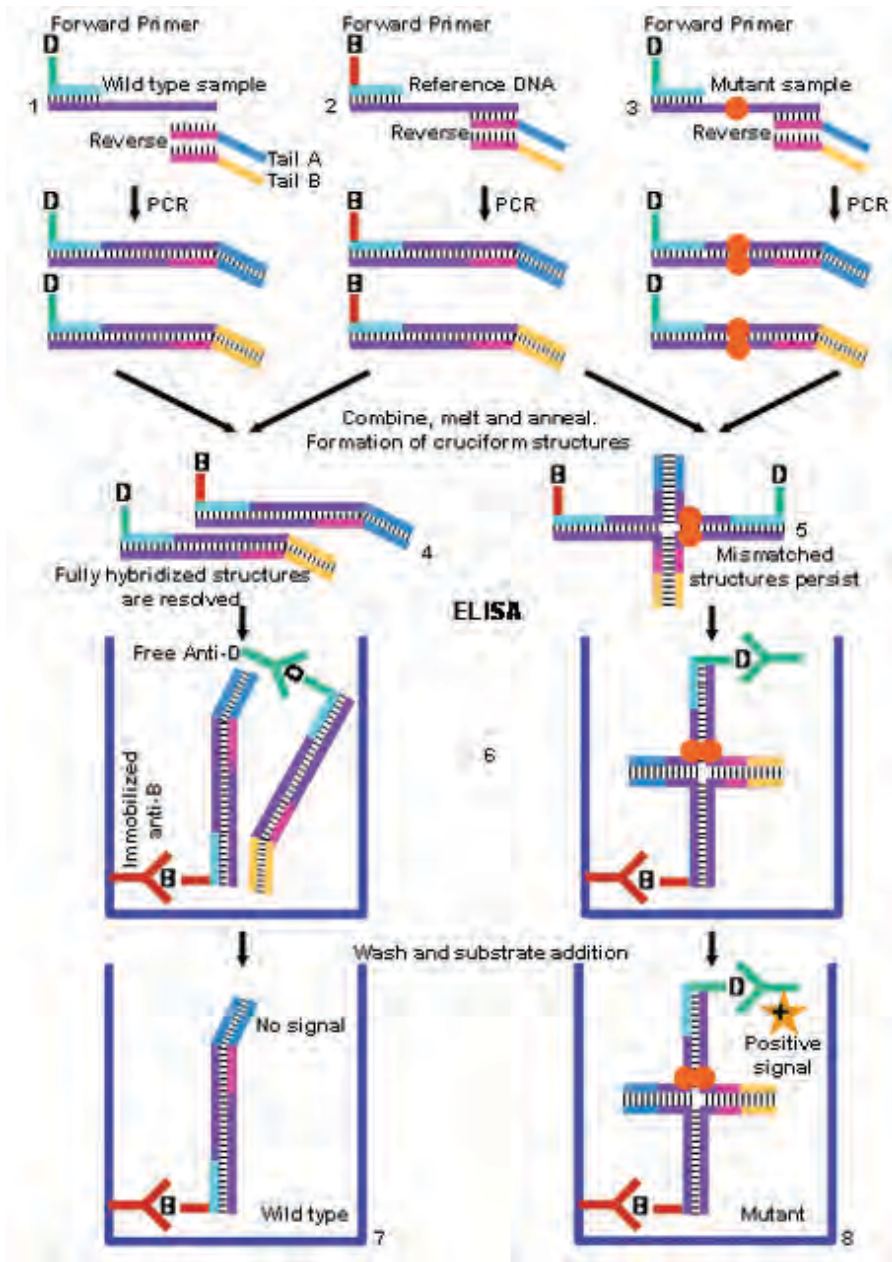
Figure 4. Simplified scheme for BMI: target sequences are amplified by PCR using a single forward primer and two tailed reverse primers (each tail with different sequences, A and B). Per assay two PCR amplifications are done: the sample is amplified using digoxigenin (D) labeled primer (1) and a reaction using a reference DNA, in which the forward primer is labeled with biotin (B) (2). The case in which the sample has a mutation is shown (3), where an orange circle indicates the mutation. PCR products of the sample and reference DNA are combined followed by denaturation and re-annealing, producing partial duplexes that assemble into four-stranded cruciform structures. These structures resolved into fully hybridized complexes when the strands have a pair of identical opposite arms (4). However, in the case the sample has a mismatch due to the presence of a mutation, the resolution of the structure is inhibited (5). Detection of the cruciform structure is achieved by a standard ELISA reaction by means of an immobilized anti-biotin monoclonal antibody and a free anti-digoxigenin monoclonal antibody (6). After washing, the ELISA reaction is developed and no signal will be observed in case of a wild type (7), or positive if mutant (8).
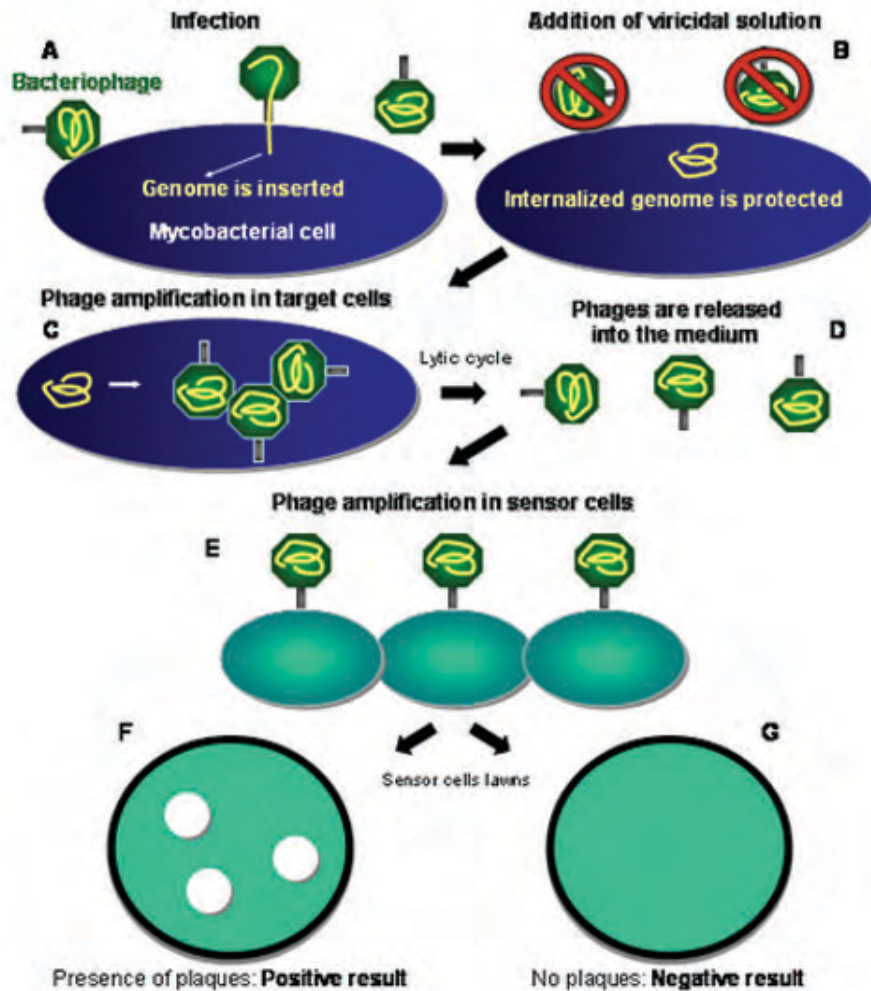
Figure 5. Detection of *M. tuberculosis* by PhaB assay. A. Phages are added to the decontaminated clinical sample. Phages will infect viable mycobacterial cells present in the solution. B. Ferrous ammonium sulphate is added (viricidal solution) to destroy all non-infecting phages. C. Phages begin to replicate leading to cell lysis and release of the new phage particles into the medium (D). E. This solution is added onto a lawn of sensor cells (*M. smegmatis*) to produce plaques (F). In case the sample was negative, no plaques will be present (G).

mutations associated to DR. Most of these techniques have promising results but have not yet been tested with statistically significant number of samples.

Several promising assays are based upon the amplification refractory mutation system (ARMS) principle. The 3'-end of a wild type PCR primer is located at the mutation site. In the case of a wild type template, the DNA polymerase will amplify the DNA efficiently, yielding a clear band using gel electrophoresis. In the case of a mutant template, the 3'-end of the primer will not hybridize and the DNA polymerase will not amplify resulting in the absence of a band by gel electrophoresis. However, in practice when there is a 3'-end mismatch a less efficient amplification can be observed, leading to background that can hinder the assay interpretation. This background could be further diminished by a stringent standardization of the PCR conditions. ARMS has been applied to different mutations in *rpoB, katG* and *embB* with promising results (59-62). These approaches offer an inexpensive alternative for
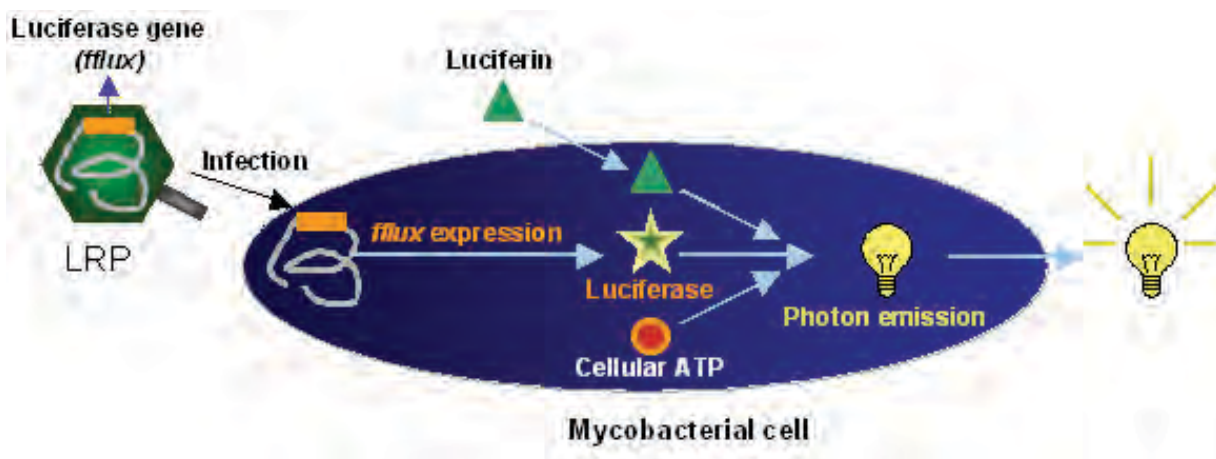
Figure 6. LRP assay principle. LRPs act as molecular vectors of the *fflux* gene (which codes for the firefly luciferase) into mycobacterial cells. LRPs are able to inject the viral chromosome specifically into mycobacterial cells. Subsequent *fflux* expression depends on the cellular machinery and luciferase activity depends on the availability of cellular ATP and the addition of luciferin (which is the luciferase substrate) to the medium. Luciferase catalyzes a reaction in which photons are released to be detected by a photographic film or a luminometer. Emission of light in a sample infected with LRPs and supplemented with luciferin indicates the presence of viable mycobacterial cells. If the sample is pretreated with antibiotics, light is detected only if resistant cells are present.

strain genotyping but their standardization is laborious with the inherent risk of amplicon contamination.

Branch Migration Inhibition (BMI) is a novel technique based on the fact that spontaneous strand exchange is inhibited by any sequence difference between two DNA molecules. It offers a simple approach to detecting all possible polymorphisms in an amplicon in a single reaction. A simplified description of the technique is shown in figure 4. For a detailed description of this technique please refer to references (63,64). In this technique two PCR reactions are performed using in one a reference DNA and in the second the sample DNA, using the same forward primer either labeled with digoxigenin or biotin respectively. In both reactions the reverse primer used has two different 5'-end tails. The PCR products are mixed, denatured and re-annealed. If the sequence is wild type a transient cruciform structure is resolved into two fully complementary primer pairs. If the sample has a mutation, the cruciform structure is more stable and can be easily detected in an ELISA reaction because it carries both labels. This technique allows screening larger regions of DNA in a single reaction

when compared with molecular beacons or FRET probes. BMI was used for detection of DR to RIF and pyrazinamide in MTB. Preliminary results are in agreement with sequencing results, but not with phenotypic analysis due to the detection of silent mutations in *rpoB* (65).

Diagnosis of TB and drug resistance with mycobacteriophages

Usage of mycobacteriophages for diagnosis of TB and DR offer a phenotype-based result in short time and low cost. This is especially important for diagnosis of DR, because results are obtained regardless of the mechanisms of DR, mutation site, with similar turnaround time of molecular approaches. Molecular techniques determine presence of specific nucleic acids, either from live or dead cells, while phages depend only on viable mycobacterial cells. There are two kinds of mycobacteriophage-based assays: the phage-amplified biological assay (phaB) and the luciferase reporter phages (LRPs).

PhaB

The phaB determines the protection and amplification of a phage by mycobacteria in clinical specimens (66,67) (figure 5). The clinical sample
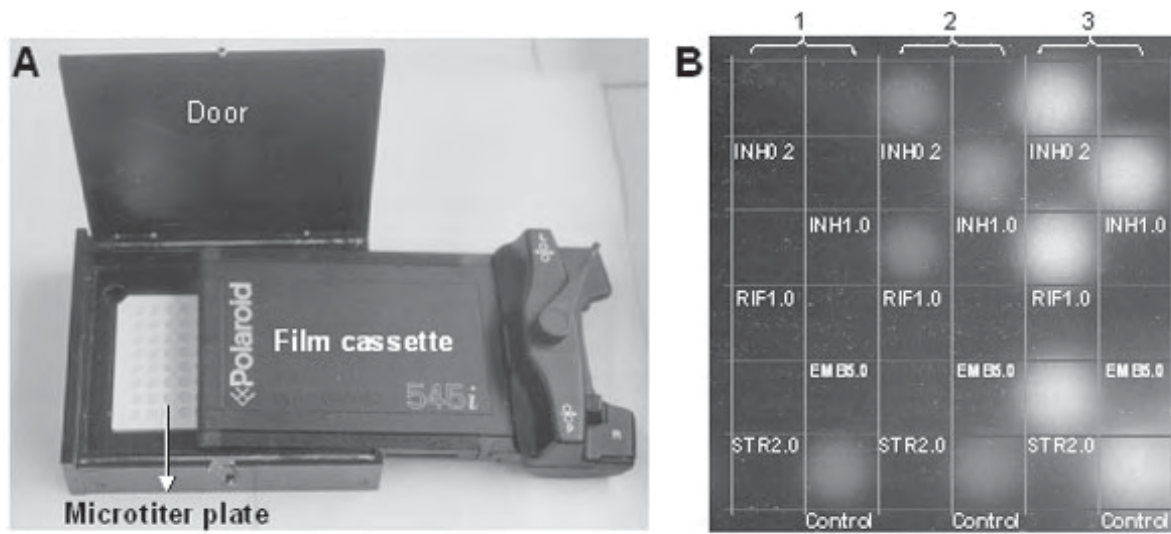
Figure 7. Bronx box. A. Picture of the Bronx box. MTB cultures after incubation with and without antibiotics are placed in microtiter plates. After 8 hours of LRP infection, a buffer with luciferin is added, the plate is sealed with a self-adhesive transparent acetate film and loaded into the Bronx box. Immediately after the film cassette is loaded over the plate, the door is closed and the overnight film exposure begins. B. Photographic detection of light emitted by susceptible MTB isolate (1) and the resistant isolates (2 and 3) incubated during 72 hours in media supplemented with INH, RIF, streptomycin (STR) and ethambutol (EMB) at the concentrations shown (μg/ml). Wells labeled as control correspond to no antibiotic treatments. Absence of signal indicates susceptibility. Results shows that isolate 1 is susceptible, isolate 2 is INH and RIF^R and isolate 3 is resistant to all antibiotics except ethambutol.

suspected of containing viable mycobacterial cells is infected with the phages. Once the infection has occurred, the cells are washed with a viricidal solution destroying all extracellular phages. The intracellular phages will replicate, lysing *M. tuberculosis* cells, and new phage particles will be released into the media. The number of particles will be proportional to the number of cells infected, which will be quantified by counting plaques using a layer of *M. smegmatis*. If sample aliquots are treated with antibiotics prior to infection with the phage, plaques will appear only if the mycobacterial cells are resistant (66-68).

For the detection of *M. tuberculosis* a commercial system has been developed, the FASTplaqueTB assay (Biotec Laboratories, South Africa) with promising results using sputum and urine samples, offering a viable diagnostic alternative for low-income countries, with a turnaround time of 1 day from sample collection (69). For the diagnosis of DR, phaB results have been satisfactory using

clinical isolates and sputum specimens (66,67,70,71).

LRPs

LRPs are phages harboring the *fflux* (firefly luciferase) gene, which produces visible light when expressed in the presence of luciferin (enzyme substrate) and cellular ATP (figure 5). LRPs are able to infect, replicate and express their genome (including the *fflux* gene) within viable mycobacterial cells. If an LRP-infected clinical sample releases light after addition of luciferin, the presence of viable *M. tuberculosis* is detected (72). If the sample is incubated with antibiotics followed by LRP infection, light emission is proportional to the resistance level (73-75). LRP assays can be used as well for the identification of species from the *M. tuberculosis* complex (72,76). Luciferase activity can be monitored with a luminometer or with photographic film. The luminometer offers higher sensitivity, quantitative results within 54 hours, simple procedure and

analysis, and has shown promising results with clinical isolates and sputum samples (76,77). Photographic detection is achieved by using the 'Bronx box' (figure 7). The Bronx box is an inexpensive light-tight box with a Polaroid cassette that carries a photographic film that is able to detect light emitted by the infection carried out in microtiter plates. The Bronx box yields qualitative results in 94 hours (78). Both techniques have shown promising results showing high agreement with BACTEC, MGIT and agar proportion method as reference (76-78).

Conclusions

The expectation that molecular techniques would surpass conventional methods for diagnosis of TB or phenotypic susceptibility testing has not (yet) been realized. The genetic basis of resistance must be understood before achieving such a goal. However, the clinician now has a variety of new tools to improve the diagnosis of TB and DR. Most of them still require detailed and systematic evaluations using standard techniques as references before their widespread application in clinical settings. Most of these techniques require trained personnel and specialized equipment, hindering their application in field conditions, but they can be used in reference laboratories as part of the TB control programs. The physician must be cautious when using results obtained by these techniques, especially when diagnosing DR. Although it is not recommended, these molecular methods might be used as a complement to the standard methods in situation of difficult diagnosis, but never should be used solely to base such decisions.

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