Palomino • Leão • Ritacco

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From Basic Science to Patient Care

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Chapter 14: New Diagnostic Methods

Enrico Tortoli and Juan Carlos Palomino

14.1. Introduction

The diagnosis of mycobacterial infections remained practically unchanged for many decades and probably would have not progressed at all without the unexpected resurgence of tuberculosis (TB) which characterized the last twenty years of the 20th century.

With microscopy lacking wide margins for improvement, the areas which most benefited from the renewed interest in TB were culture and identification, while a completely new approach emerged, aimed towards the direct detection of mycobacterial nucleic acids in clinical specimens.

14.2. Automated culture methods

Although known for decades, liquid media for cultivation of mycobacteria had never attracted the attention of mycobacteriologists. In fact, the ability of a liquid medium to support a faster growth was heavily hampered by its susceptibility to contamination. The use of antimicrobial combinations suitable of inhibiting the growth of the whole spectrum of potential contaminants (Gram-positive and Gram-negative bacteria as well as fungi) represented a turning point.

During the same period, automation was taking its first steps in microbiology, with blood cultures leading the field. The apparently banal idea of exporting such technology to mycobacterial cultures evolved into selective liquid media, which were a[0] breakthrough for diagnostic mycobacteriology.

14.2.1. BACTEC TB-460

The BACTEC TB-460 system (Becton Dickinson, Sparks, MD) was the first, and for many years the only, automated approach in mycobacteriology. It makes use of a radiometric instrumentation developed for blood cultures with the broth bottles replaced by vials containing a medium specific for mycobacteria.

The principle

The medium: A modified Middlebrook 7H9 medium is used, in which one of the components, palmitic acid, is radiolabeled with ^{14}C. Contamination is controlled by the addition, prior to use, of a mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin (PANTA) reconstituted with a poly-oxyethylene solution. The use of such a combination of antibiotics does not eliminate the decontamination step, which needs to be performed before inoculation of the samples. The vials containing the medium remain sealed through the whole culture process and the specimen is inoculated by puncturing the rubber septum with a needle (Figure 14-1).

Figure 14-1: Inoculation of a BACTEC vial

The instrumentation: Once the paired needles have perforated the rubber septum of the vial, the gaseous phase is aspirated and replaced with air containing 5 % CO_2. The aspirated gas is analyzed by a β-counter to quantify the eventual presence of radiolabeled CO_2 (Figure 14-2).

The rationale: When viable mycobacteria are present in the culture vial, the radiolabeled palmitic acid is metabolized and radioactive CO_2 is liberated into the gaseous phase.

The performance

The BACTEC TB-460 was first commercialized in 1980 and soon became popular worldwide. It is not a fully automated system, as the vials, which are held in an

external incubator, must be loaded into the instrument for reading. The reading is usually performed twice a week during the first 15 days of incubation, and weekly thereafter, until the 42nd day. BACTEC TB-460 is still used in many laboratories worldwide but its glorious course is starting to wane. The increasing cost of radioactive waste disposal and the interest of the manufacturer to promote newly-developed alternative systems are slowly prevailing over its still excellent performance.

Figure 14-2: The BACTEC TB-460 instrument

From the first evaluations, BACTEC TB-460 revealed a clear superiority over solid media systems in terms of sensitivity and time to detection of positive culture (Middlebrook 1977, Damato 1983, Morgan 1983, Takahashi 1983, Park 1984, Siddiqi 1984). Many years later, despite the release of new systems, the BACTEC radiometric method is still competitive (Alcaide 2000, Brunello 1999, Laverdiere 2000, Piersimoni 2001, Badak 1996, Ganeswrie 2004, Scarparo 2002, Tortoli 1998, Tortoli 1999). The BACTEC TB-460 system is also suitable for mycobacterial blood culture using designated bottles.

14.2.2. BACTEC MGIT960

The BACTEC MGIT960 system (Becton Dickinson, Sparks MD) uses the technology of the previously developed blood culture instrument. The original system (BACTEC 9000) was first adjusted to support mycobacterial cultures but was sub-

sequently completely redesigned to process tubes, which are much less cumbersome to handle than the original bottles.

The principle

The medium: Mycobacteria Growth Indicator Tube (MGIT) is a modified Middlebrook 7H9 medium in which a supplement is added at the moment of use. The supplement is a mixture of oleic acid, albumin, dextrose, and catalase (OADC) enrichment and the same PANTA antibiotic mixture used in the radiometric system. The presence of PANTA does not do away with the decontamination step, which needs to be done before inoculation. As the tubes containing the medium are screw-capped, no needle is needed for inoculation. A silicon film embedded with a ruthenium salt is present at the bottom of the tube as a fluorescence indicator (Figure 14-3).

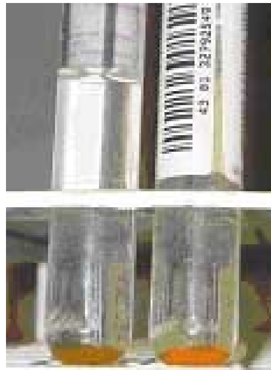


Figure 14-3: MGIT tube

The instrumentation: Incubator and reader are combined in a single cabinet (Figure 14-4). The bottom of each tube, stimulated by ultraviolet light, is monitored by a fluorescence reader. Fluorescence-emitting tubes are reported as positive. It is also possible to use the MGIT tubes without instrumentation, by holding the tubes in a normal incubator and observing the fluorescence under a Wood's lamp.

The rationale: The oxygen normally present in the medium quenches the natural fluorescence of the ruthenium salt. If viable mycobacteria are present in the tube, oxygen is consumed due to their metabolism, the quenching effect lowers accordingly, and the bottom of the tube fluoresces when exposed to ultraviolet light.

Figure 14-4: The MGIT960 instrument

The performance

The BACTEC MGIT960 is a typical walk-away instrumentation which monitors the tubes at one-hour intervals, alerts when they become positive and signals the end of the incubation period.

Many studies have evaluated the BACTEC MGIT960 in comparison with similar competitor systems and also with solid media. BACTEC MGIT960 turned out to be clearly faster and more sensitive than solid media, while the comparison with other automated and semi-automated systems revealed substantially overlapping performance (Alcaide 2000, Badak 1996, Scarparo 2002, Tortoli 1999, Casal 1997, Pfyffer 1997, Tortoli 1997). Mycobacterial blood cultures cannot be performed with this system.

14.2.3. VersaTREK

The VersaTREK (previously known as the ESP system II) uses the technology of a previously developed blood culture system and is commercialized by Trek Diagnostic Systems.

The principle

The medium: It uses a modified Middlebrook 7H9 medium to which the OADC enrichment must be added. Two different antimicrobial mixtures are available. The first one, also known as AS, includes polymyxin B, azlocillin, fosfomycin, nalidixic acid, and amphotericin B. The second contains polymyxin B, vancomycin, nalidixic acid, and amphotericin B (PVNA). Usually, AS is used for specimens originating from sterile samples or with a low risk of contamination, while PVNA is used for heavily-contaminated samples. The presence of such antimicrobial mixtures for contamination control does not eliminate the decontamination step, which needs to be performed before inoculating the sample. The bottles of medium (Figure 14-5) hold a cellulose sponge whose large surface area allegedly improves growth. Bottles are inoculated through a rubber septum by means of a syringe.

Figure 14-5: VersaTREK bottle (*Courtesy of TREK Diagnostic Systems*)

The instrumentation: Incubator and reader are combined in a single cabinet (Figure 14-6), which also shakes the bottles during the incubation. The pressure within each bottle is monitored by a manometer through a proper connector. Cultures presenting a decreased headspace pressure are reported as positive.

The rationale: If viable mycobacteria are present in the bottle, the oxygen consumption due to their metabolism reduces the internal pressure.

The performance

VersaTREK is a typical walk-away instrumentation which continuously monitors the bottles, alerts when they become positive and signals the end of the incubation period.

Several studies have evaluated VersaTREK in comparison with solid media and similar automated and semi-automated competitor systems. VersaTREK clearly performs better than solid media but shows no substantial advantage over other systems (Tortoli 1998, Williams-Bouyer 2000, Woods 1997).

Figure 14-6: The VersaTREK instrument (*Courtesy of TREK Diagnostic Systems*)

Mycobacterial blood cultures can also be performed with the VersaTREK system. However, whole blood cannot be used and a previous treatment is required to obtain sediment for inoculation. Either a buffy-coat or sediment obtained with the lysis-centrifugation method is suitable to inoculate the bottles. The lysis-centrifugation method (Isolator, Oxoid, United Kingdom) consists of saponin-containing tubes to lyse blood cells, a proper centrifugation procedure, and special pipettes for elimination of supernatant and collection of the sediment.

14.2.4. BacT/Alert 3D

BacT/Alert 3D (previously known as MB/BacT) is commercialized by bioMérieux and uses the technology of a previously developed blood culture system.

The principle

The medium: A modified Middlebrook 7H9 medium is used in which a supplement, a mixture of OADC enrichment and polymyxin B, amphotericin B, nalidixic acid, trimethoprim, vancomycin and azlocillin, is added at the moment of use. The presence of such contamination-controlling antibiotics does not eliminate the decontamination step needed before inoculation. The bottles of medium have a CO_2 sensor at the bottom and are inoculated through a rubber septum by means of a syringe.

The instrumentation: Incubator and reader are combined in a single machine (Figure 14-7) which does not shake the bottles during incubation.

Figure 14-7: The BacT/Alert system

The CO_2 sensor is impacted by a light whose reflected ray is monitored by a photodiode (Figure 14-8). Bottles producing specific changes in the intensity of the reflected light are reported as positive.

The rationale: If viable mycobacteria are present in the bottle, the CO_2 produced by their metabolism causes a change in the color of the sensor, from green to yellow, which alters the intensity of the reflected light ray (Figure 14-9).

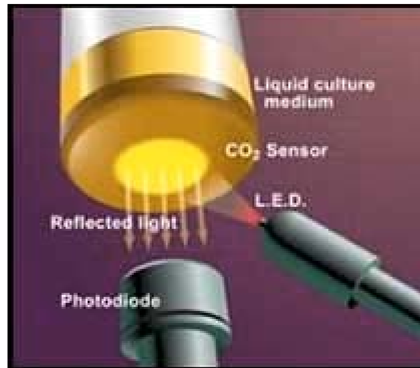


Figure 14-8: The BacT/Alert technology

Figure 14-9: Negative and positive bottles

The performance

BacT/ALERT 3D is a typical walk-away instrumentation which monitors the bottles at 10-min intervals, alerts when they become positive, and signals the end of the incubation period.

Many studies have evaluated the BacT/ALERT 3D. The system turned out to be clearly faster and more sensitive than conventional media, while the comparison with other automated and semi-automated systems did not reveal significant differences (Alcaide 2000, Brunello 1999, Laverdiere 2000, Nogales 1999, Piersimoni 2001, Roggenkamp 2000, Rohner 1997, Saito 2000, Yan 2000).

The system is also suitable for mycobacterial blood cultures, provided proper bottles are used; no previous treatment of the blood is required.

14.3. Nucleic acid amplification methods

When the polymerase chain reaction (PCR) methodology took its first steps into diagnostic microbiology, a restricted number of micro-organisms appeared to have the potential to benefit from the novel technique. *M. tuberculosis* was among them, and the dream of the rapid diagnosis of TB appeared to be about to come true.

14.3.1. *In house* methods for diagnosis of tuberculosis

One of the first findings on the way to developing a PCR technique (Figure 14-10) aimed at *M. tuberculosis* detection was that, although different targets were investigated, none of them were suitable for differentiating *M. tuberculosis* from other species belonging to the *M. tuberculosis* complex. Such limitation, due to the extremely high genome similarity (close to 100 %) among the members of the *M. tuberculosis* complex, did not, however, cool the enthusiasm. In fact, the differentiation of such species is of very limited relevance from the clinical and therapeutic point of view.

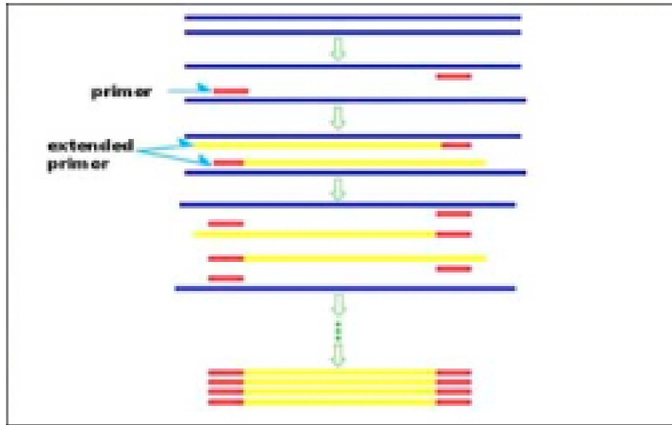


Figure 14-10: PCR

Among the first proposed genomic targets for diagnostic PCR was the newly detected insertion element IS*6110* which, being present in multiple copies (from four to 20 in more than 95 % of *M. tuberculosis* strains), appeared to have the potential for an enhanced sensitivity (Figure 14-11). Other successfully used desoxyribonucleic acid (DNA) regions include the 65 kiloDalton (kDa) heat-shock protein gene, the gene encoding the 126 kDa fusion protein, and the gene encoding the β-subunit of ribonucleic acid (RNA) polymerase; all of them are present in single copies in *M. tuberculosis* complex genomes.

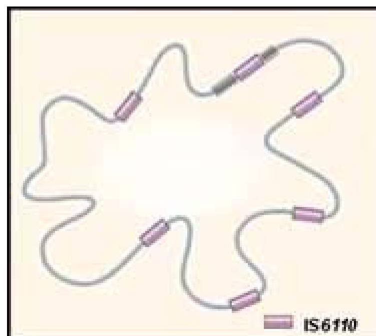


Figure 14-11: *M. tuberculosis* genome with six copies of IS*6110*

In the '90s, many laboratories developed various *in house* PCR protocols and soon the aspiration of an increased sensitivity led to the adoption of "nested" PCR. In this procedure, the amplification of a large region of DNA is followed by a second amplification targeted to a shorter internal stretch. Most of such *in house* PCR procedures achieved a sensitivity never matched by commercial systems but were often burdened by the high incidence of false positive results due to amplicon cross-contamination of specimens.

In subsequent years, with the purpose of avoiding the PCR patent, alternative amplification methods were developed. Most successful were the reverse transcriptase PCR, the ligase chain reaction, and the strand displacement amplification.

14.3.2. Commercial methods

In the last few years, several amplification methods have been commercialized; only four methods have gained worldwide diffusion and been widely validated by international studies, although one of them (LCx, Abbott) is no longer on the market.

Amplified MTD

Amplified *Mycobacterium tuberculosis* Direct Test (AMTD), developed by Gen-Probe (San Diego, CA, USA), is an isothermal (42°C) transcriptase-mediated amplification system.

The principle

A *M. tuberculosis* complex-specific region of the 16S ribosomal RNA gene produces double-stranded ribosomal DNA, due to the combined action of reverse-transcriptase and ribonuclease. In turn, RNA polymerase catalyzes the synthesis of multiple stretches of ribosomal RNA from the ribosomal DNA synthesized before. A new cycle starts when the newly produced ribosomal RNA undergoes further transcription by reverse transcriptase (Figure 14-12).

The sensitivity of the method is increased by the presence, in each bacterium, of a high number of 16S ribosomal RNA target molecules (about 2,000) compared to only one copy of 16S ribosomal DNA. Another advantage of the amplification from RNA relies on the low stability of such a molecule; this minimizes both the risk of contamination and the incidence of false-positive results due to the persistency of stable nucleic acids (DNA) in the host organism, even after the complete eradication of the infection.

The detection of amplification products relies on hybridization with a specific, single-strand DNA probe labeled with a chemiluminescent molecule (Hybridization Protection Assay).

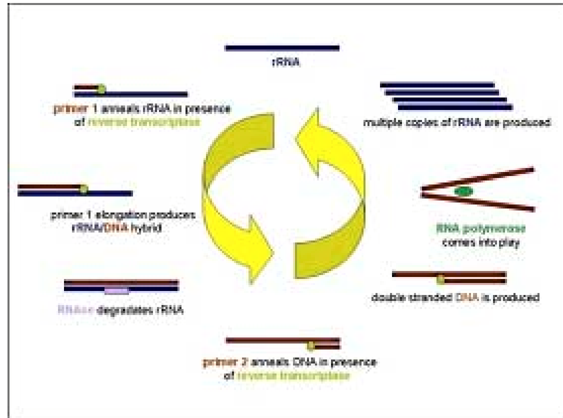


Figure 14-12: The cycle of the transcriptase-mediated amplification

The features

The whole process is performed manually, starting with the extraction by means of sonication, continuing with the addition of different reagents until the final reading with the luminometer (Figure 14-13). Thermal-cyclers are not needed and the whole amplification step is carried out on a heating block at 42°C. The turnaround time is 2.5 hours. No internal control is provided in the kit to monitor the presence of inhibitors.

The method is approved by the Food and Drug Administration of the United States of America (US FDA) for testing smear-positive and smear-negative respiratory samples.

Figure 14-13: The luminometer

The performance

From a review of the huge amount of literature available, sensitivity ranging from 91.7 % to 100 % in smear-positive samples and from 65.5 % to 92.9 % in acid fast bacilli (AFB) smear-negative samples has been reported (Alcala 2001, Chedore 1999, Chedore 2002, Gamboa 1998, O'Sullivan 2002, Piersimoni 2003, Woods 2001). To reduce the prevalence of false-positive results, an equivocal zone in the interpretation of results has been recently introduced with the recommendation of retesting samples scoring within this range (Kerleguer 2003, Middleton 2002).

Amplicor MTB Test

The Amplicor MTB Test (Roche Molecular Systems, Basel, Switzerland) relies on standard PCR.

The principle

A 584 bp fragment of the 16S ribosomal RNA gene, comprising a species-specific region flanked by genus-specific sequences, is amplified using biotinylated primers. In the master mix, an unusual combination of nucleotides is present – as an adjunct to adenine, guanidine and cytosine, uracil is used in place of thymine. As a consequence, the amplification product differs from the target DNA in that it contains uracil instead of thymine. This device is part of a contamination-control system based on the use of uracil-N-glycosylase, an enzyme that fragments DNA wherever uracil is present. The enzyme, added to the samples before amplification,

destroys any amplicon resulting from previous amplifications without damaging the uracil-free target DNA. Because of the genus-specific nature of the annealing regions, 16S ribosomal DNA belonging to any mycobacterial species is amplified by this PCR. The use, in the revealing phase, of magnetic beads coated with *M. tuberculosis* complex-specific probes allows the removal, by washing, of any other DNA. The detection of the specific amplification product is performed by adding an avidin-enzyme conjugate and a chromogenic substrate.

The features

The amplification and detection steps are carried out automatically by the Cobas Amplicor instrument (Figure 14-14). Once the sample extraction has been performed by heating (95°C), the tube is placed in the thermal cycler integrated in the Cobas instrument. Without further handling, the amplification product will be automatically transferred into the detection station where the chromogenic reaction is developed and read. The turnaround time is 6-7 hours. The method is approved by the US FDA for testing smear-positive respiratory samples. It includes an internal control, composed of synthetic DNA characterized by identical annealing sequences as the mycobacterial target; when this is not amplified, it signals the presence of inhibitors. The detection of *M. tuberculosis* complex DNA can also be carried out without the Cobas instrument, using a manual kit that, however, does not include an internal control.

Other Amplicor kits are available for detection of *Mycobacterium avium* and *Mycobacterium intracellulare* DNA in clinical samples.

Figure 14-14: The Cobas Amplicor instrumentation

The performance

From the literature review, specificity is close to 100 % while sensitivity ranges from 90 % to 100 % in smear-positive samples and from 50 % to 95.9 % in smear-negative ones (Bogard 2001, Eing 1998, Gamboa 1997, Gomez-Pastrana 2000, Mitarai 2000, Piersimoni 2003, Rajalahti 1998, Reischl 1998, Shah 1998).

BD ProbeTec ET

The BD ProbeTec ET (Becton Dickinson, Sparks, MD) uses DNA polymerase and isothermal strand displacement amplification to produce multiple copies of IS*6110*, an insertion element unique to *M. tuberculosis* complex.

The principle

The rationale of strand displacement amplification is extremely complex; what is presented here is an extreme simplification. In the initial phase (target amplification), amplification is started by two pairs of primers complementary to contiguous sequences delimiting the target. The elongation of the upstream primer, also named bumper, determines the displacement of the simultaneously elongating downstream primer and finally releases the produced amplicon. A restriction site, present in the downstream primer, will also be present in the released amplicon (Figure 14-15A). In the exponential amplification phase, a new primer anneals to the amplicon and, following digestion by the restriction enzyme, the upstream fragment acts as bumper and displaces the downstream fragment (Figure 14-15B).

Real-time detection is based on the energy transfer technology. A hair-pin-shaped probe, complementary to IS*6110*, is marked by two fluorescent molecules, one of which, the donor, is quenched by the other, the acceptor; furthermore, it presents a restriction site in the sequence between the two markers (Figure 14-16A). Once its free end has hybridized with the amplification product, the probe undergoes elongation (Figure 14-16BC) before being displaced by a primer annealed upstream to the same amplicon (Figure 14-16D). The elongation makes the probe able to bind a new primer (Figure 14-16E) which, while elongating, stretches out the “hair-pin” and moves the acceptor away from the donor (Figure 14-16FGH). The nicking of the restriction site by a proper enzyme further separates donor and acceptor and allows the first to free a fluorescence signal (Figure 14-16I).

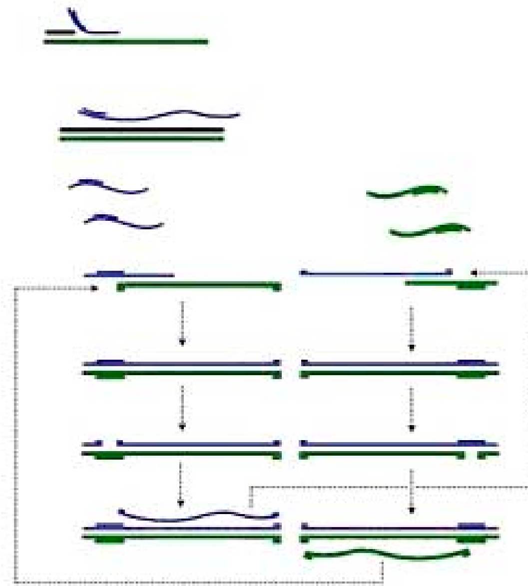


Figure 14-15: ProbeTec ET amplification cycles

The features

Some manipulation is required before introduction of the sample into the automatic instrument (Figure 14-17); each sample is first inactivated at 105°C, and then sonicated to extract the DNA, transferred into a priming well at 72.5°C, and subsequently into an amplification well at 54°C. In the BD ProbeTec ET instrument, the microplate containing the samples and the amplification reagents is incubated at 52.5°C and the fluorescence emitted is continuously monitored. A thermal cycler is not required. The turnaround time is 3.5 to 4 hours.

An internal control is present, characterized by the same annealing sequences as the mycobacterial target. In case of amplification failure, this control alerts for the presence of inhibitors.

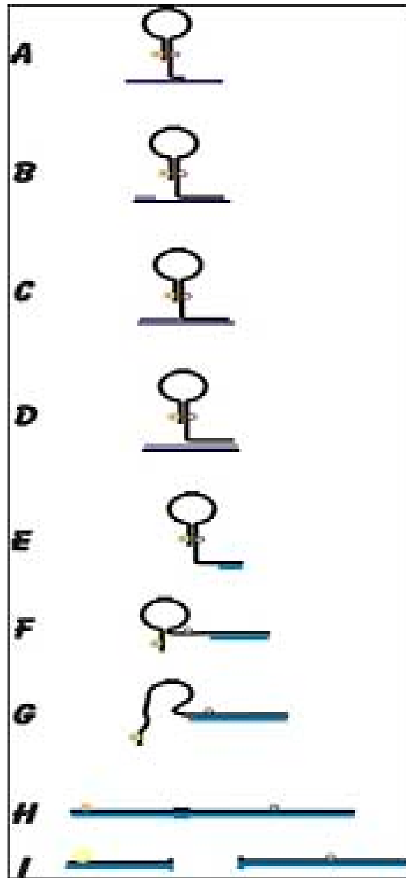


Figure 14-16: The detection step of ProbeTec ET

The system is not yet approved by the US FDA.

Kits are also available for the amplification of nucleic acids of *M. avium*, *M. intracellulare* and *Mycobacterium kansasii*.

The performance

The literature reports a rate of sensitivity ranging from 98.5 % to 100 % for smear-positive samples and very variable (0.33 %-100 %) for smear-negative ones (Barrett 2002, Bergmann 1998, Bergmann 2000, Johansen 2002, Maugein 2002, Mazzarelli 2003, Pfyffer 1998, Piersimoni 2002).

Figure 14-17: The BD ProbeTec ET instrument

14.3.3. Comments on amplification methods

Although direct amplification methods are used worldwide, they are far from having revolutionized clinical mycobacteriology. Culture, supported by microscopy, still remains the gold standard, and molecular methods only represent a useful support in some cases, to speed up the diagnosis of TB.

The unsatisfactory sensitivity is the major limitation of amplification-based methods. It is now evident that paucibacillary specimens have little chance of being detected by molecular amplification. Factors that contribute to the reduction of the sensitivity are the uneven distribution of bacilli in the sample, the suboptimal extraction of nucleic acids, and sometimes the presence of inhibitors. The phenol-chloroform extraction unquestionably provides the best yield but, being cumbersome and time consuming, also raises the risk of contamination. To minimize this risk and to make the technique user friendly, the commercial systems have probably oversimplified this step by reducing it to sonication or heat treatment only. The sediment of a number of samples contains substances inhibiting the amplification process. The reason for their presence is unknown and at present there is no known method for neutralizing them. The use of an internal control represents a major feature to be taken into account at the moment of choosing an amplification method.

Although the specificity of amplification methods is substantially good, the possibility of false-positive results should be borne in mind by both microbiologists and clinicians. A therapy like the one against TB, which is long and not exempt from side-effects, should not be undertaken only on the basis of a positive result of a single amplification test. The major reason for false-positive nucleic acid amplification results is the contamination of samples, possibly in the pre-analytic, but mostly in the analytic phase. The application of dedicated procedures, such as the one employing uracil-N-glycosylase or the adoption of sealed amplification chambers, is useful. More important still are general precautions such as the frequent decontamination of the work environment with 10 % bleach and the exposure of pipettes, tips and bench surfaces to ultraviolet light when not in use. Finally, a major role is played by the training and the expertise of the operator.

A particular category of false-positive results is that concerning samples obtained from patients under treatment. In these patients, the detectability of mycobacterial DNA over a long period of time, despite the effectiveness of the treatment, is well known and clearly makes DNA amplification useless for treatment monitoring.

The above limitations and recommendations have also been stressed by the US Centers for Disease Control and Prevention (CDC, detailed information on the internet at http://www.cdc.gov/mmwr/preview/mmwrhtml/mm4926a3.htm) (Figure 14-18). The observance of such recommendations is extremely important. However, the CDC's advice against the use of commercial PCR methods with non-respiratory samples may appear outdated nowadays. In recent years, a number of articles have been published showing that the amplification methods can be of use for extrapulmonary specimens too, although impaired by lower sensitivity. Still, their limitations should be kept in mind and a system with internal control should be used due to the high frequency of inhibitors (Alcala 2001, Chedore 1999, Chedore 2002, Eing 1998, Gamboa 1998, Johansen 2002, Maugein 2002, Mazzarelli 2003, O'Sullivan 2002, Reischl 1998, Rimek 2002, Woods 2001). An obvious, but often disregarded, point is that the lower sensitivity is not, in the large majority of cases, due to the extrapulmonary origin but to the lower bacterial load inherent to such samples.

Among the impressive amount of publications assessing different amplification methods and the few studies concerning direct comparisons (Della-Latta 1998, Piersimoni 2002, Scarparo 2000), none convincingly demonstrates the superiority of one over the others. All are characterized by equally good specificity and insufficient sensitivity. The substantially similar performance of all systems makes it difficult to understand the different rating given by the FDA to the systems available on the market.

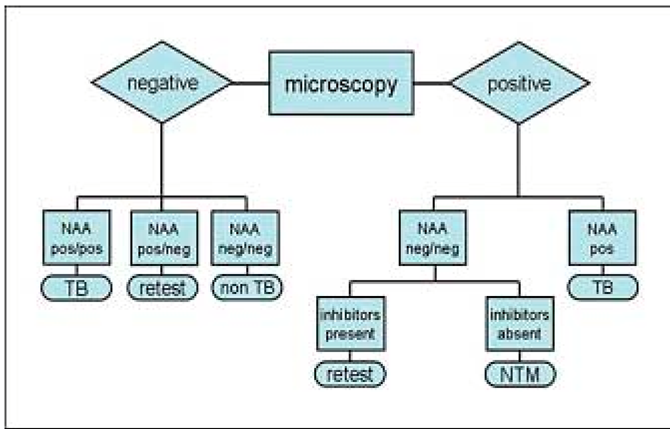


Figure 14-18: CDC recommendations for interpretation of nucleic acid amplification tests

14.3.4. Future prospects

Real-time PCR now seems to be on the point of being adapted for the diagnosis of TB. The evident delay at this step, in contrast with its well established use in other fields of diagnostic microbiology, suggests the emergence of some problems. Major expectations are concerned with the increase in sensitivity, while the availability of quantitative results may represent the first step towards its use for treatment monitoring.

Nevertheless, despite its evident usefulness and potential improvements, it seems unlikely that nucleic acid amplification can replace culture for diagnosis of TB in the short term. Culturing is still essential for monitoring the response to therapy and testing antimicrobial susceptibility.

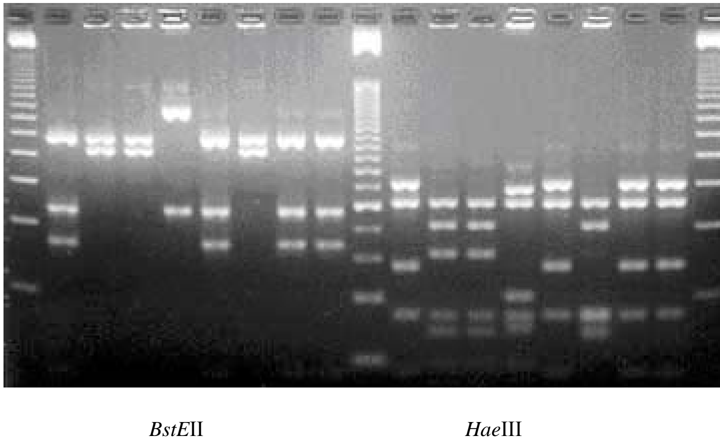
14.4. Genetic identification methods

Following the extraordinary development of molecular methods, the identification of mycobacteria, previously based on phenotypic investigations, suddenly started to rely on genotypic methods. Different genetic approaches developed in research laboratories became rapidly popular in diagnostic laboratories and some of them were transformed into commercial diagnostic kits.

14.4.1. PCR restriction-enzyme analysis

The principle

The PCR restriction-enzyme analysis (PRA) method is based on the amplification of a 441-bp fragment of the *hsp65* gene by PCR, followed by the digestion of the amplified product with two restriction enzymes *BstE*II and *Hae*III according to the procedure first described by Telenti (Telenti 1993). The products of the digestion reaction are then separated and visualized by agarose gel electrophoresis (Figure 14-19). The restriction pattern thus obtained is compared to an algorithm present in the PRASITE available on the internet at http://app.chuv.ch/prasite/index.html. This database comprises 74 PRA patterns corresponding to 38 defined species of mycobacteria. In recent years, several novel PRA patterns from newly characterized species have been described. The PRA method has also been used with other genomic regions for the identification of mycobacteria such as the *rpoB* and *gyrB* genes with good results (Lee 2000, Goh 2006).



*BstE*II *Hae*III

Figure 14-19: PRA patterns of different species of mycobacteria (*Courtesy S. Leão*)

The features

The PRA method can be applied on heat-inactivated and washed bacterial suspensions obtained from mycobacteria grown either on solid or in liquid medium. There is only one report on the direct application of PRA to clinical samples (Magalhaes

2002). Being an *in house* method, and when properly standardized, it is a convenient alternative to more costly commercial identification methods.

The performance

There are many studies on the application of the PRA-*hsp65* method for the rapid identification of mycobacteria. In general, the method has proved to be practical, cost-effective, and highly accurate for mycobacterial identification. In a recent multicenter evaluation, performed in eight laboratories in Latin America that received a set of coded strains for identification, the PRA-*hsp65* method proved to be highly accurate and easy to perform. The accuracy of the identification can be further improved when combined with minimal microbiological characteristics such as growth rate and pigmentation. Nevertheless, attention should be paid to a few technical details such as gel preparation and running, and some training is needed in the interpretation of patterns (Leão 2005).

14.4.2. DNA probes

The DNA-probe technology for identification of fastidious organisms is still one of the most successful molecular diagnostic procedures worldwide. In this sense, the role played by commercial DNA probes in the quality improvement of mycobacterial identification in clinical laboratories cannot be disregarded.

AccuProbe

The precursor system, AccuProbe (Gen-Probe, San Diego, CA), was developed almost 20 years ago. It is still very popular, mainly due to its extremely simple procedure. Indeed, it is the only DNA-probe system not requiring previous amplification of the target.

The principle

The probe is a single-stranded DNA oligonucleotide, complementary to a short, species-specific sequence within a hypervariable region of the 16S ribosomal DNA. It is labeled with an acridinium ester, a chemiluminescent molecule, which gives light when properly excited. Once the mycobacterial cell has been lysed by sonication, the extract is mixed with the probe under stringent conditions, allowing their hybridization only in case they are 100 % complementary.

As the chemiluminescent marker, easily accessible in the native probe, turns out to be protected in the double-stranded hybrid (Hybridization Protection Assay), the

addition of a hydrolyzing agent makes the first undetectable without affecting the second. Any hybridization is accompanied by light emission, which is detected with a luminometer, thus simplifying the identification of the test strain.

The features

Different AccuProbe kits are available for identifying mycobacteria belonging to the *M. tuberculosis* complex, the *M. avium* complex and the species *M. kansasii*, *Mycobacterium gordonae*, *M. avium* and *M. intracellulare*. They can be used on cultures grown either on solid or in liquid medium. The only equipment required is a sonicator for cell lysis and a luminometer (Figure 14-13) for the final reading.

The performance

The sensitivity and specificity of AccuProbe are widely acknowledged in the literature (Bull 1992, Drake 1987, Enns 1987, Gonzales 1987, Kiehn 1987, Lebrun 1992, Musial 1988, Saito 1989, Tortoli 1994, Tortoli 1996). Only a few unspecific reactions have been reported, mainly for the *M. avium* complex probe, which also hybridizes with the recently described species *M. palustre* (Torkko 2002), *M. parascrofulaceum*, and *M. saskatchewanense* (Turenne 2004).

Line probe assays

The line-probe assay uses the reverse hybridization technology with differently-specific DNA-probes immobilized in parallel lines on a paper strip. The target DNA, previously extracted by boiling, is PCR-amplified using biotinylated primers and finally incubated with the strip. Once the hybridization has been carried out under highly stringent conditions and the unbound amplicons have been washed out, the hybridized probe is revealed as a colored band, developed following the addition of a streptavidin-labeled enzyme and a chromogenic substrate (Figure 14-20). The specificity of the hybridized line-probe is inferred by the position of the colored band on the strip. Three commercial methods are available, INNO-LiPA MYCOBACTERIA (Innogenetics, Ghent, Belgium), GenoType Mycobacterium (Hain, Germany), and GenoType MTBC (Hain, Germany).

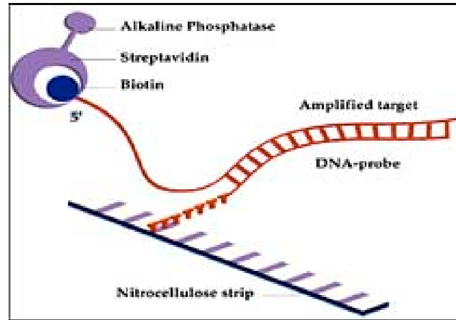


Figure 14-20: Line Probe assay (*Courtesy of Innogenetics N.V.*)

INNO LiPA Mycobacteria

The line probes of INNO LiPA Mycobacteria are species-specific fragments of the internal transcribed spacer (ITS) region interposed between 16S and 23S ribosomal RNA genes. The system includes a genus *Mycobacterium*-specific probe, two complex-specific probes (*M. tuberculosis* complex and *M. avium* complex) and 23 other probes suitable for identifying 18 species and several intra-specific variants (within the *Mycobacterium chelonae-abscessus* group and in species *M. kansasii*) (Figure 14-21). A thermal cycler and a shaking water bath are needed; an automated instrumentation is also available to carry out the hybridization step (Figure 14-22).

Figure 14-21: Inno-LiPA interpretation

Figure 14-22: Auto-LiPA instrument

GenoType Mycobacterium

The line probes of GenoType Mycobacterium are fragments of the 23S ribosomal RNA gene mostly shared by more than one species. In this case, the identification is not based on the specificity of a single line but on the different combinations of multiple bands characterizing each species (Figure 14-23). Different patterns are suitable for ascertaining if the test strain belongs to a group of related genera characterized by high guanosine plus cytosine content, to the genus *Mycobacterium*, to the *M. tuberculosis* complex or to any of 35 mycobacterial species. Furthermore, it distinguishes two intra-specific variants within the species *M. fortuitum*. The system is available as two kits which are sold separately. One of them, GenoType CM, identifies the more frequently detected mycobacterial species with 17 line-probes, while the other, GenoType AS, includes 18 probes aimed at the less common species.

A thermal cycler and a shaking water bath are needed; an automated instrumentation is also available to carry out the hybridization step.

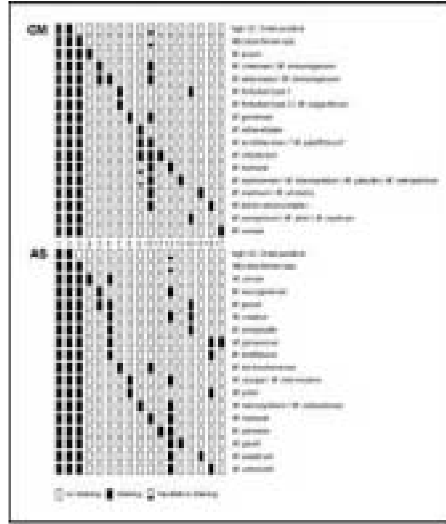


Figure 14-23: GenoType Mycobacterium interpretation chart

GenoType MTBC

The newly developed GenoType MTBC is a reverse hybridization system devoted to the identification of the species belonging to the *M. tuberculosis* complex which cannot be differentiated by the analysis of any of the most frequently investigated conserved regions (16S ribosomal DNA, ITS, 23S ribosomal DNA). In this kit, multiple genetic regions are targeted in a multiplex PCR assay. Eleven probes are present on the strip: one is aimed at the 23S ribosomal DNA, nine at four regions of the *gyrB* gene, and one at the flanking regions of RD1. The 23S ribosomal DNA-specific probe is used to confirm the isolate as belonging to the *M. tuberculosis* complex.

The hybridization patterns of the nine probes aimed at different regions of the *gyrB* gene, in which single-nucleotide mutations may be present, differentiate *M. tuberculosis*, *Mycobacterium africanum* type I, *Mycobacterium bovis*, *Mycobacterium caprae,* and *Mycobacterium microti*; they cannot, however, distinguish *M. tuberculosis* from *M. africanum* type II and from "*Mycobacterium canettii*". The differentiation of *M. bovis* from *M. bovis* BCG, which is not feasible on the basis of *gyrB* mutations, is obtained with the last probe suited to detect the deletion of RD1 characterizing *M. bovis* BCG. This probe, which is complementary to the two genetic regions delimiting RD1, can, in fact, only hybridize if RD1 is missing, as is the

case in BCG (Figure 14-24). A thermal-cycler and a shaking water bath are required; automated instrumentation is also available to carry out the hybridization step.

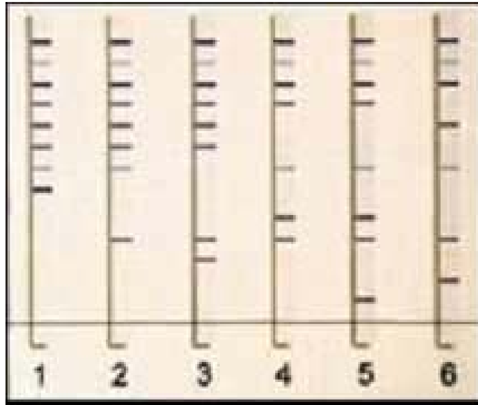


Figure 14-24: GenoType MTBC interpretation: 1, *M. tuberculosis*; 2, *M. africanum* type I; 3, *M. microti*; 4, *M. bovis*; 5, *M. bovis* BCG; 6, *M. caprae (Courtesy of HAIN Lifescience)*

The performance

Line-probe assays represent an important progress in DNA-probe technology since they allow the simultaneous testing of the organism with a number of probes. Specificity and sensitivity are high (70-80 %) The only cross reactions reported so far concern rarely encountered rapidly growing mycobacteria (Tortoli 2001, Tortoli 2003) or species not previously described at the moment at which the probes were developed (Tortoli 2005, Tortoli 2006). A limitation of GenoType is the presence of a number of equivocal hybridization patterns that are shared by two or more species due to a moderate variability of the 23S ribosomal DNA (Figure 14-23).

14.4.3. Genetic sequencing

The targets

Every genetic region which is highly conserved and, at the same time, includes moderately variable sequences is a potential target for identification. A number of such regions are known in the genome of living organisms; among them, the best

known include several genes: 16S ribosomal DNA, 23S ribosomal DNA, *hsp65,* and the non-encoding stretch ITS.

The 16S ribosomal DNA, which is about 1,500 bp long, is by far the most popular target for sequencing, and the one for which the largest database is available. In this gene, universal sequences shared by practically every living organism co-exist with genus-specific sequences common to the organisms belonging to the same genus (e.g. the genus *Mycobacterium*) and with species-specific sequences that differentiate between species. With regard to the mycobacteria, almost all the sequences characterized by species-specific variability are concentrated in the first third of the gene (Figure 14-25), namely in two stretches called hypervariable region A and hypervariable region B. The first covers nucleotides between positions 130 and 210, and the second includes nucleotides from position 430 to position 500 (such numbers indicate the corresponding positions within the *Escherichia coli* 16S ribosomal RNA gene) (Rogall 1990a, Rogall 1990b). A practical consequence is that the determination of the nucleotide sequence of the first 500 bp of the gene allows the differentiation of almost all mycobacterial species known at present.

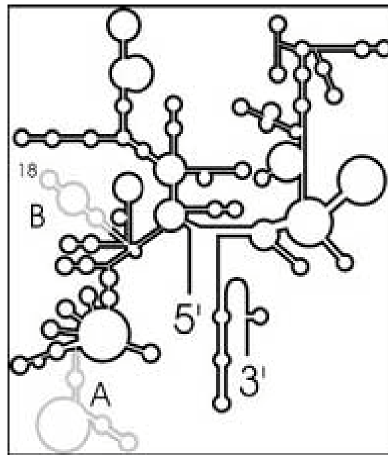


Figure 14-25: The 16S ribosomal RNA

The principle

Genetic sequencing is nowadays performed using automatic sequencers that render it highly reproducible (Figure 14-26). Initially, the target region is PCR-amplified

using proper primers and standard nucleotides. Then, the amplification product, once denatured, undergoes a second amplification in which the 3' and the 5' primers are used in separate tubes. In this step, in addition to standard nucleotides, a lower proportion of special nucleotides terminating the chain elongation are present. The random incorporation of terminator nucleotides during the amplification procedure (the four bases are marked with a different fluorochrome) produces strands whose lengths range from that of the primer elongated by a single nucleotide to that of the whole target. The amplicons, each marked with the fluorochrome specific for the nucleotide with which it ends, are put in order of length by means of electrophoresis. The emerging patterns of fluorescent markers identify the last bases of a continuous series of stretches, each being one nucleotide longer than the previous one, and consequently determine the genetic sequence of the region.

It is very important to perform the sequencing of both 3' (forward) and 5' (reverse) strands, to carefully resolve the discrepancies.

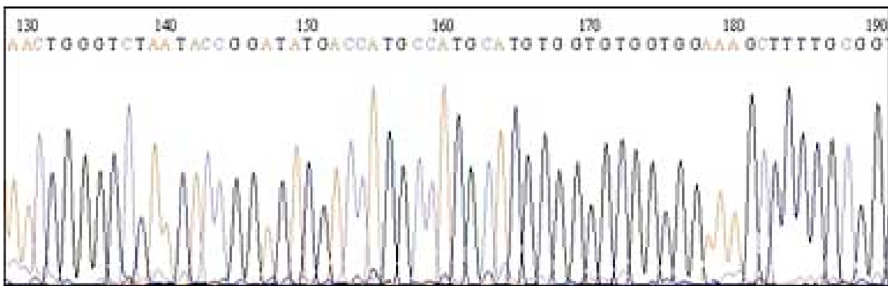


Figure 14-26: Automatic sequencing: the electropherogram

The databases

Once a sequence has been determined, its comparison with known sequences is required. Several databases are available on the Internet; fortunately, the most popular exchange new submissions made to any of them. These databases are GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html), EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/) and the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/). The available sequences in such databases are continuously updated by new submissions from the users. This feature is at the same time a strength and a weakness. In fact, the control of submissions is very soft and the presence of short fragments (sequences as long as 50 bp are accepted), or of

sequences determined in the years in which the sequencing technique was still dawning, turn out to be misleading (Turenne 2001).

Ribosomal Differentiation of Medical Microorganisms web-server is a public-domain database (RIDOM: http://www.ridom-rdna.de/) limited to sequences of the 16S ribosomal DNA and the 16S-23S ITS region. Different from the previous ones, it is strictly controlled and does not allow submissions by users. Unfortunately, the value of RIDOM, which indeed represents an ambitious innovation, has been hindered by the total lack of updating in the last years.

The performance

Unquestionably, genetic sequencing is nowadays the reference identification method, not only for mycobacteria but for all microorganisms, and the 16S ribosomal DNA is still the most important target sequence. Sequencing of the 5' end (about 500 bp) provides final results for the vast majority of members of the genus *Mycobacterium*. The determination of the full gene is needed to distinguish *M. peregrinum* from *M. septicum* (Schinsky 2000, Tortoli 2003), *M. murale* from *M. tokaiense*, *M. marinum* from *M. ulcerans* (Kirschner 1993), and *M. novocastrense* from *M. flavescens* sqv. ii. The only species that cannot be distinguished from each other on the basis of 16S ribosomal DNA are *M. kansasii* from *M. gastri* (Böddinghaus 1990), *M. mucogenicum* from *M. phocaicum* (Adékambi 2006), *M. fluoranthenivorans* from *M. hackensackense* (Tortoli 2006), and lastly, *M. abscessus*, *M. massiliense* (Aékambi 2004) and *M. bollettii* from each other (Adékambi 2006).

Due to its wider variability (its length ranges from 270 to 400 bp), ITS (Roth 1998) can be usefully sequenced to differentiate the rapidly growing species, which are more closely related to each other than the slow growers. Rapid growers have two copies of the ribosomal operon (except for *M. chelonae* and *M. abscessus* which have one) and a single organism may possess two different ITS copies. This may make the interpretation of the electropherograms problematic because of the presence of overlapping peaks. To obviate this problem, cloning of the genetic region is required before sequencing.

Another increasingly used genetic target for identification purposes is a 440 bp sequence of the 65 kDa heat shock protein gene, whose length is 1,623 bp. The corresponding database has almost been completed in the last few years (McNabb 2004). As is the case with the ITS region, this gene shows much higher variability than the 16S ribosomal DNA (McNabb 2004, Ringuet 1999).

Among other sequence targets for mycobacterial differentiation, the most important include the genes *recA* (Blackwood 2000), involved in DNA repair, *sodA* (Bull

1995) encoding for superoxide dismutase, and *rpoB* encoding for the beta-subunit of RNA-polymerase. The latter, which includes highly variable regions and is present in a single copy in all mycobacteria, has been recently proposed as the gold standard for the differentiation of rapidly growing mycobacteria (Adékambi 2003).

Although the minimal standards concerning genetic analysis have not been defined, it is universally agreed that at least the sequence of the full 16S ribosomal DNA gene must be determined for the description of a sp. nov.

14.5. Non-conventional phenotypic diagnostic methods

In addition to the so-called conventional methods for TB diagnosis and besides the automated and molecular diagnostic methods described above, some new technologies have been proposed, such as phage-based assays and rapid detection of growth by microscopic observation of microcolonies in solid or liquid media.

14.5.1. Phage-based assays

The phage-based assay relies on the ability of *M. tuberculosis* to support the growth of an infecting mycobacteriophage. The number of endogenous phages, representing the original number of viable bacilli, is then determined in a lawn of a rapidly growing mycobacterium such as *M. smegmatis* (McNerney 2001). Several studies have been performed to assess the *FASTPlaque TB* assay, a commercial test based on this technology, for the early detection of *M. tuberculosis* (Albert 2002). In a comparative study with auramine smear microscopy and culture in Löwenstein-Jensen medium in 1,692 sputum specimens, it was found that the *FASTPlaque TB* test detected TB in 75 % of culture-confirmed cases and in 70 % of cases with a clinical diagnosis of TB with a specificity of 98.7 % and 99.0 %, respectively. On the other hand, the concentrated auramine smear microscopy had a sensitivity of 63.4 % and 61.3 % and a specificity of 97.4 % and 97.3 % in culture-confirmed and clinically-confirmed cases, respectively. In another study done in Pakistan, the *FASTPlaque TB* compared to acid-fast smear microscopy and culture in Löwenstein-Jensen medium had a sensitivity and specificity of 87.4 % and 88.2 %, respectively, in smear-positive specimens, and a sensitivity and specificity of 67.1 % and 98.4 %, respectively, in smear-negative samples (Muzaffar 2002). As a conclusion of these studies, the *FASTPlaque TB* was able to detect mycobacteria in 50-65 % of smear-negative specimens with a specificity of 98 %, and a combination of the test with smear microscopy confirmed the presence of *M. tuberculosis* in 80-90 % of culture-positive specimens. However, *FASTPlaque TB* failed to detect

about 13 % of the smear-positive specimens and 8 % to 19 % of the smear-negative samples gave a false-positive result (Takiff 2002).

An interesting study that compared the original in-house method with the *FAST-Plaque TB* found that neither method was able to outperform direct microscopy in sputum samples while contamination rates of 40 % were obtained with the *FAST-Plaque TB* test (Mbulo 2004). Recent modifications to this commercial system include incorporation of an antibiotic mixture to decrease the high rate of contamination.

Some other phage-based technologies using reporter mycobacteriophages have also been proposed for the rapid detection and identification of *M. tuberculosis*; however, they have not been thoroughly evaluated in clinical settings of highly-endemic countries (Carriere 1997, Banaiee 2001).

14.5.2. The micro-colony method

The micro-colony method or thin-layer agar technique is an old method for culturing and identifying mycobacteria; it allows both rapid detection and presumptive identification of isolates based on the characteristic morphology of mycobacteria in culture, and has been proposed as an inexpensive alternative method for the rapid detection and culture of mycobacteria (Welch 1993). A few years ago, Mejia *et al.* described a procedure based on this method for the rapid detection of *M. tuberculosis* microcolonies isolated from clinical samples and observed under a standard microscope. The Thin Layer 7H11 agar (TL7H11) allowed the detection of more than 60 % of the culture-positive samples within the first ten days and more than 80 % after two weeks of incubation compared to 10 % on Löwenstein-Jensen medium (Mejía 1999). In a report comprising more than 1,800 clinical samples, the same authors showed a sensitivity of 72 % for TL7H11 as compared to standard cultivation in Löwenstein-Jensen medium and concluded that the simultaneous use of both media increased the sensitivity of detection (Mejía 2004).

In a further validation of the method in different settings, the TL7H11 was evaluated in a phase II prospective multicenter study performed in six laboratories in different countries in Latin America (Robledo 2006). A total of 1,118 sputum and extrapulmonary specimens were studied. All smear-positive samples yielded positive cultures, while smear-negative samples yielded *M. tuberculosis* in 3.2 % of Löwenstein-Jensen medium cultures compared to 4.4 % by TL7H11. Sensitivity was 92.6 % (95 % CI 87.9-95.9) for TL7H11 and 84.7 % (95 % CI 78.8-89.0) for Löwenstein-Jensen medium with a median time to detection of 11.5 days (95 % CI

9.3-15.0) for TL7H11 and 30.5 days (95 % CI 26.9-39.0) for Löwenstein-Jensen medium. The reported contamination rate was 5.1 % for TL7H11 and 3.0 % for Löwenstein-Jensen medium. Taking into consideration the different characteristics and implementation conditions of the participating laboratories, the TL7H11 proved to be robust enough to enter into further evaluations and cost-effectiveness studies. Figure 14-27 below shows the typical microcolony morphology of *M. tuberculosis* after several days of incubation.

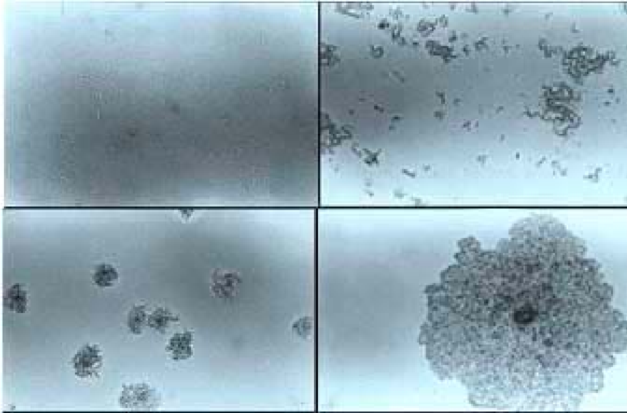


Figure 14-27: Microcolonies of *M. tuberculosis* after, 4, 6, 8, and 15 days of culture (*Courtesy J. Robledo*)

14.5.3. Microscopic observation broth-drug susceptibility assay (MODS)

MODS has been described for the early detection of *M. tuberculosis* growth in liquid medium, allowing a more timely diagnosis and drug susceptibility testing. The method is based on the observation of the characteristic cord formation of *M. tuberculosis* visualized microscopically in liquid medium with the use of an inverted microscope (Caviedes 2000). In this study, sputum samples were analyzed by staining, cultivation, and PCR. Sensitivity of MODS (92 %) compared favorably with the most sensitive of the other culture methods (93 %) with a median turnaround time of nine days. The method has been proposed as a rapid, inexpensive, sensitive, and specific method for *M. tuberculosis* detection and susceptibility testing, appropriate for use in developing countries.

In a recent operational study conducted in Peru, the performance of the MODS assay was investigated for the rapid diagnosis of TB (Moore 2006). The assay was compared with an automated mycobacterial culture system and culture on Löwenstein-Jensen medium. The sensitivity for the detection of *M. tuberculosis* was 97.8 % compared to 89.0 % for the automated mycobacterial culture, and 84.0 % for Löwenstein-Jensen medium ($P < 0.001$); the median turnaround time was 7, 13, and 26 days for MODS, the automated culture system, and Löwenstein-Jensen medium, respectively ($P < 0.001$). One limitation of the MODS assay is the requirement for an inverted microscope, which is necessary to observe the cord formation in liquid medium.

14.5.4. Analysis of cell wall mycolic acids

Mycobacteria have an unusually high lipid content in their cell wall. Such lipids include mycolic acids and other saturated and unsaturated fatty acids. Mycolic acids are branched, long-chain fatty acids present in the cell wall of a limited number of genera; they exhibit the maximum length in the genus *Mycobacterium* (Table 14-1).

Seven types of mycolic acids, differing mainly in the presence of functional groups, are variously combined in the cell wall of different species of the genus *Mycobacterium.* These types are: alpha-, alpha'-, methoxy-, keto-, epoxy, wax esters, and omega' methoxy-mycolates.

Table 14-1: Mycolic acid-containing genera

Genus	Chain length (carbon atoms)
Corynebacterium	22-38
Rhodococcus	34-52
Nocardia	44-60
Gordonia	48-66
Tsukamurella	67-78
Mycobacterium	60-90

The analysis of the lipid content of the mycobacterial cell wall has been widely used for identification purposes. The various techniques used are based on the

physical partitioning between two phases (stationary and mobile) of single lipids present in the mycobacterial cell wall.

The extraction of the lipids from the bacterial colonies is the preliminary step in all the techniques described below.

Thin-layer chromatography (TLC)

The principle

TLC uses silica plates (stationary phase) on the surface of which the mycolic acids extracted from the mycobacterial strain are separated as a result of their different affinity for a solvent (mobile phase), advancing by capillarity. Once the plate has been stained, each species displays a particular dot pattern according to its mycolic acid content that can be identified by comparison with patterns of reference strains with known mycolic acid composition run in parallel (Minnikin 1975) (Figure 14-28).

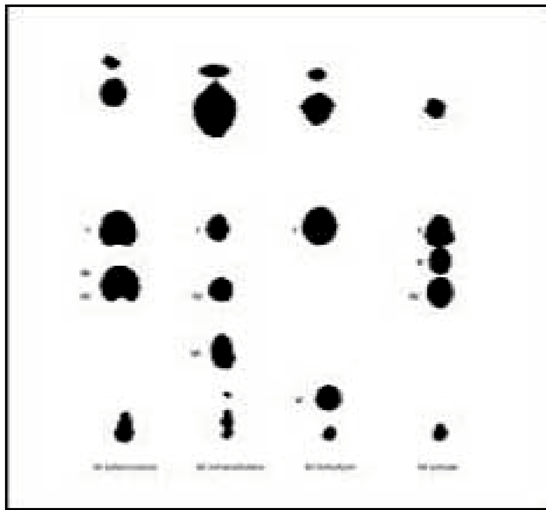


Figure 14-28: TLC of mycolic acids

The limitations

The high number of mycobacterial species, already over 130, has substantially scaled down the relevance of TLC for identification at the species level. With only seven types of mycolic acids and with most mycobacteria including no more than two or three of them, the number of TLC patterns shared by more than one, and often by many, species is high.

Gas-Liquid Chromatography (GLC)

The principle

In GLC, a gas (mobile phase) is used to carry the sample through a liquid (stationary phase) contained in a column. Once the lipids extracted from a mycobacterial strain have been injected, the high operating temperature of the column (about 300°C) produces the cleavage of the mycolic acids in saturated methyl esters 22-, 24- and 26-carbon atoms long. Along with such mycolic acid fragments, saturated and unsaturated fatty acids (including tuberculostearic acid) and alcohols are eluted.

The recognition of different elution products is usually obtained by mass spectrometry. Cleavage products, which are unvaried within single mycolic acid types, produce, along with fatty acids and alcohols, patterns consistent to single species and suitable for their differentiation (Guerrant 1981, Lambert 1986, Larsson 1985) (Figure 14-29).

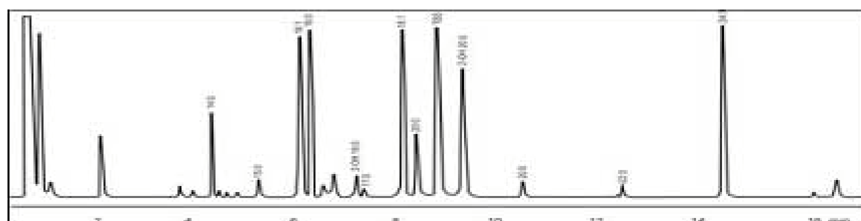


Figure 14-29: GLC pattern of *M. avium*

The limitations

The major problems concerning the GLC are the limited inter-laboratory reproducibility and the insufficient discriminative power related to the high number of *Mycobacterium* species.

High-Performance Liquid Chromatography (HPLC)

The principle

The HPLC uses high pressure to carry a liquid (mobile phase) containing the extracted sample, through the particulate (stationary phase) present in the column. The various types of mycolic acids, previously saponified, extracted and derivatized to bromophenacyl esters, are separated in the column and eluted at different times. On the basis of individual ultraviolet absorbance, the detector plots single fractions as peaks arranged in a profile. The profile of each species is sufficiently different from those of other species (Figure 14-30) to provide identification when visually compared with profiles of known mycobacteria (Butler 1988, Butler 1991, Butler 2001, Tortoli 1996). A fluorescence-based detection system may also be used that is more sensitive than the ultraviolet-based system.

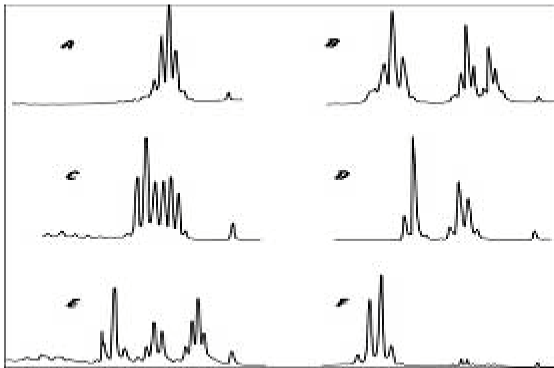


Figure 14-30: HPLC Representative patterns: A, *M. tuberculosis complex,* B*, M. intracellulare;* C*, M. gordonae;* D*, M. chelonae;* E*, M. simiae;* F*, M. nonchromogenicum*

The limitations

For many years, HPLC has been considered the only phenotypic method suitable for differentiating almost all mycobacterial species. The number of species hardly differentiable or not distinguishable at all, which was insignificant until a few years ago, has recently increased, due to the continuous description of new species, in particular, of rapidly growing mycobacteria.

A wide library of HPLC profiles, including more than 100 mycobacterial species, is available on the internet at http://www.mycobactoscana.it/page4.htm.

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