# Mycobacteria

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## Diagnostics

It is universally accepted that mycobacterioses due to nontuberculous mycobacteria are acquired from the environment, and patients with acquired immunodeficiency syndrome (AIDS) are not an exception. In contrast with *Mycobacterium tuberculosis*, environmental mycobacteria are weakly virulent and may require various predisposing conditions in order to cause disease in humans. The weakening of immune defenses, particularly of the cell-mediated variety, which reaches its maximum expression in the late stages of AIDS, is certainly the most important of such conditions.

Since mycobacterial colonization of the gastrointestinal tract is usually followed in AIDS patients by dissemination through the bowel mucosa, early detection of these infection is important. Stools and intestinal biopsies are the major sources of information in such investigations, the aim being either to detect mycobacteria microscopically and by means of cultures, or to reveal the presence of their components, both somatic and genomic.

### Collection of Specimens

Stool specimens of at least 1 g should be collected in sterile disposable containers and sent immediately to the laboratory. Biopsy specimens, taken aseptically, should be sent to the laboratory in saline within sterile disposable containers. No fixative should be used.

### Microscopy

An important feature of mycobacteria is acid-fastness. Indeed, once these organisms have been stained, they do not release the dye even when treated with various acids and alcohols. Using a special staining procedure it is thus possible to distinguish mycobacteria from all other microorganisms.

A smear may be made directly from unprocessed fecal specimens, but biopsy tissues must be previously ground in a homogenizer.

The smear is prepared by scraping a loopful of the specimen on a small area of the slide (about 2×1 cm), and leaving it to dry in a biological safety cabinet. The dry slides are heat fixed by passing them three or four times through the blue cone of a Bunsen flame or by placing them on an electric warmer at about 70°C for at least 2 h.

The most widely used are the Ziehl-Neelsen and the fluorochrome staining procedures.

In the Ziehl-Neelsen method, the smear is flooded for 5 min with phenolic fuchsin (0.3 g of basic fuchsin dissolved in 10 ml of 95% ethanol and added to 95 ml of aqueous 5% phenol), the slide is heated with a Bunsen burner to steaming, rinsed with tap water until no more stain comes off, and then flooded with an acid-alcohol solution (3 ml of concentrated hydrochloric acid mixed with 97 ml of 95% ethanol). The final steps include a further wash with tap water, counterstain by flooding the smear with methylene blue (3% aqueous solution) for 30 s, a final wash with tap water, and, after drainage, air drying. Under a 100× oil immersion objective, the mycobacteria appear on the slide as red-stained rods on a blue background (Fig. 1).

In the fluorescent staining procedure, the slide is covered for 15 min with auramine (0.1 g of auramine O dissolved in 10 ml of 95% ethanol and 90 ml of 3% aqueous phenol) and then rinsed with tap water and drained. A decolorizing step is performed with acid-alcohol solution (0.5 ml of concentrated hydrochloric acid mixed with 100 ml of 70% ethanol) for 2 min followed by washing and draining. After flooding for 2 min with potassium permanganate (5% in water), the slide is rinsed, drained, and air dried. When the smear is examined with a 20× to 40× objective in a fluorescence microscope, the mycobacteria appear as yellow fluorescing rods against a dark background (Fig. 2).

Auramine stain is more sensitive than the fuchsin, but reading by well-trained personnel is required.

Microscopy lacks sensitivity. Only mycobacterial loads over $2–5\times10^3$ acid-fast bacilli per milliliter of sample will be detected [1], and there is no ability to distinguish the various species of the genus *Mycobacterium* and to detect mixed infections. It is, however,

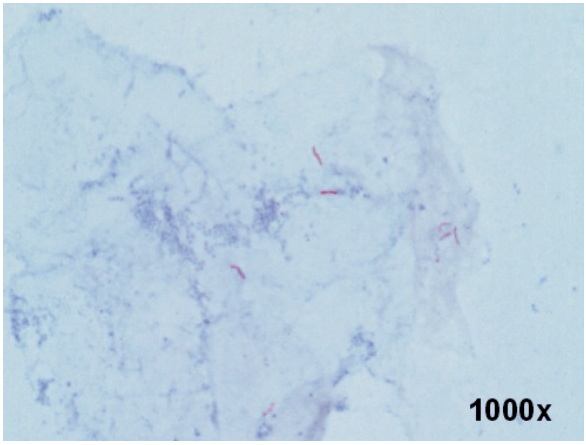


**Fig. 1.** Tubercle bacilli stained according to Ziehl-Neelsen method

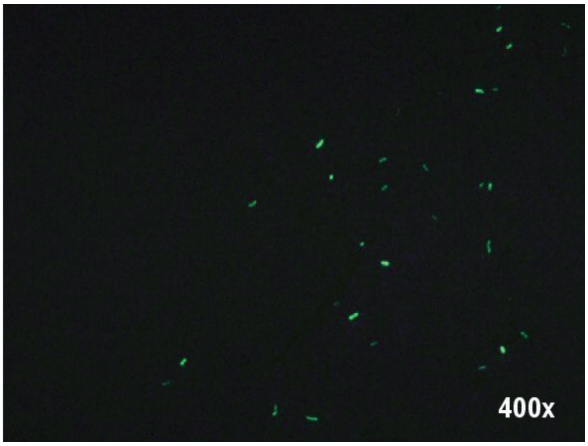


**Fig. 2.** Fluorescence (auramine O) staining of tubercle bacilli

a very reliable, inexpensive, and rapid technique whose use in combination with culture still represents the backbone of modern mycobacteriology. In many developing countries, microscopy remains the only method available for diagnosis of tuberculosis. In consideration of its importance, it is recommended that the result of acid-fast stains be reported within 24 h of specimen collection.

## Isolation of Mycobacteria

The most common media for isolation of mycobacteria are those that are egg-based (and the most widely used of these is the Lowenstein-Jensen preparation). However, a long time is required for the growth of mycobacteria, and incubation should be carried through 8 weeks. Selective solid media incorporating antibiotics may also be used, mainly for culture of heavily contaminated specimens. Agar-based media are also used, both plain and selective, which allow a slightly more rapid recovery and a better survey of morphology.

When using liquid media, particularly with specimens originating from nonsterile body sites, addition of drug blends which make them selective is mandatory.

The radiometric Bactec system (Becton-Dickinson, Sparks, Md., USA) [2], which is based on the production of radioactive $CO_2$ from a radio-labeled substrate, represents a milestone in diagnostic mycobacteriology and is now unanimously considered the gold standard, being characterized by the highest sensitivity and the shortest times to recovery.

Recently, several fully automatic culture systems, based on investigation of different metabolic activities, have been developed which challenge the radiometric system. They all combine very good performances with the elimination of radioactivity. Among them the Bactec 960 (Becton-Dickinson) makes use of the development of fluorescence, when metabolically active organisms are present in the medium, by an oxygen sensor present at the bottom of the tube [3]. The ESP II system (Trek Diagnostics, Westlake, Ohio, USA) detects living mycobacteria by the reduction of pressure due to their oxygen consumption [4]. Finally, BacT/Alert 3D system (Organon Teknika, Turnhout, Belgium) detects viable organisms by using a colorimetric sensor of produced $CO_2$ and reflected light technology [5].

Comparable results are also achievable with nonautomated liquid methods MGIT (Fig. 3) (Becton-Dickinson) [6], MB-Redox (Fig. 4) (Biotest, Dreieich, Germany) [7], or with the biphasic Septi-Chek AFB system (Becton-Dickinson) [8, 9].

The culture of stools for mycobacteria was for

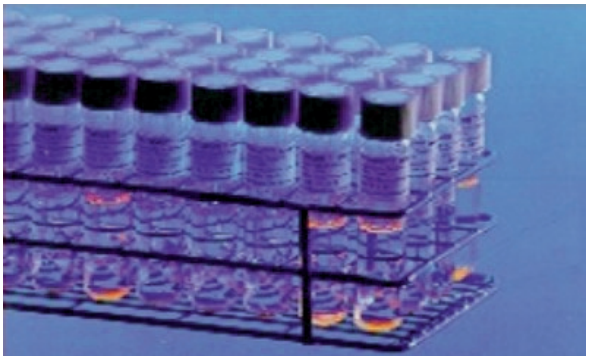

**Fig. 3.** Mycobacterial growth indicator tube, liquid medium with fluorescence growth detection

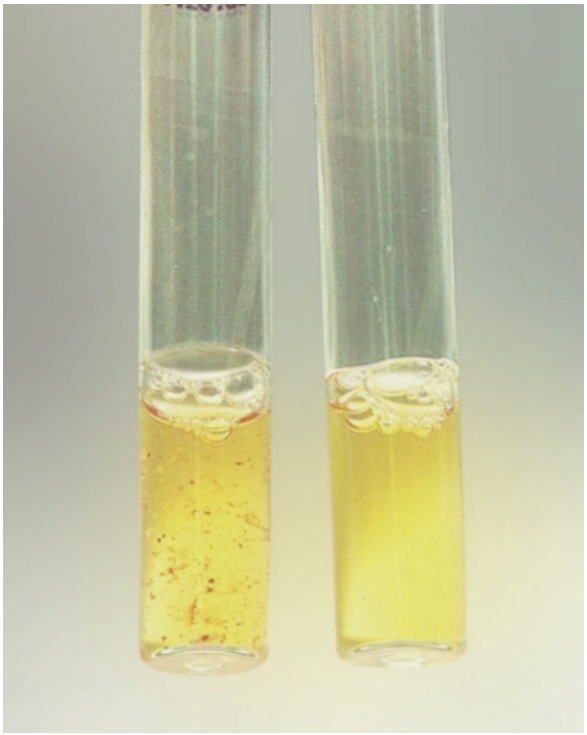

**Fig. 4.** Positive and negative MB Redox, liquid medium with tetrazole reduction-based growth detection

many years recommended only when acid-fast bacilli were detected in the direct smear. Several studies have demonstrated, however, that the sensitivity of smears from stools is no higher than 35% [10, 11]; therefore determination of whether a mycobacterial culture is to be done should no longer be based on microscopy.

Being heavily contaminated, stools require a decontamination procedure more harsh than for pulmonary specimens; a modification of the classic Petroff's sodium hydroxide method [12] is considered suitable. The fecal sample is dissolved in an equal volume of 4% sodium hydroxide in a 50-ml screw-cap centrifuge tube; after agitation and 20-min incubation at room temperature, phosphate buffer (pH 6.8) is added up to 50 ml and mixed well. The tube is then centrifuged at ≥3,000 *g* for 15 min, the supernatant is decanted, and the pH of the sediment is checked with a few drops of phenol red indicator; when necessary the sediment is neutralized with 2N hydrochloric acid. The sediment is then resuspended in 1–2 ml of distilled water and used to inoculate appropriate culture media.

The use of selective solid media is advisable but, to avoid the possibility of losing fastidious mycobacteria, simultaneous inoculation of a liquid medium is suggested.

Biopsy specimens usually do not require decontamination, but tissues coming from the intestinal tract are not sterile and need to be treated. A procedure similar to that described for stools using 2% sodium hydroxide may be suitable. As generally recommended for other mycobacterial cultures, the inoculation of a solid and a liquid medium is advisable.

Incubation of egg-based tubes at 37°C should be protracted, for at least 1 week, in a slanted position and with the screw-cap loosened, preferably in a 5%–10% $CO_2$ atmosphere, and then continued with a tightened cap (special atmosphere no longer necessary). Agar-based plates need 5%–10% $CO_2$ and, to avoid desiccation, should be incubated in $CO_2$-permeable polyethylene bags.

Inoculated media are subsequently examined visually at weekly intervals to detect the colonies of mycobacteria and contaminants as soon as they appear; for better observation of agar plates, a stereomicroscope or a common microscope with a 10× objective may be used (Fig. 5). An 8-week incubation at 37°C is usually recommended for solid media, while 6 weeks are generally considered enough for liquid media. The possible presence of an extremely slow-growing organism such as *Mycobacterium genavense* in the gastrointestinal tract of AIDS patients suggests that cultures in both solid and liquid media should be incubated for 8 weeks in these individuals [13].

The time needed for growth in culture varies with the species and depends on the bacterial load in the sample. We may expect, for instance, *Mycobacterium avium* to require 7–20 days in liquid media, and usually more than 1 month on solid egg-based media (Figs. 6–8). Although liquid media almost always provide the most rapid detection times, their use in combination with solid media is recommended to

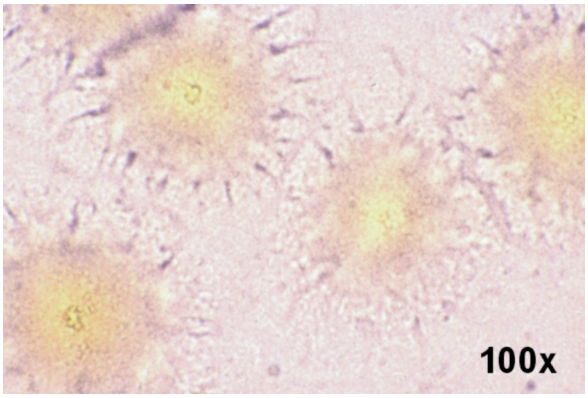


**Fig. 5.** *Mycobacterium avium* complex, opaque microcolonies

**Fig. 6.** *Mycobacterium avium* complex, growth on Lowenstein-Jensen

**Fig. 7.** *Mycobacterium tuberculosis*, growth on Lowenstein-Jensen

**Fig. 8.** *Mycobacterium kansasii*, growth on Lowenstein-Jensen

speed the identification of the strain and for the detection of mixed cultures.

The isolation of nontuberculous mycobacteria from potentially colonized sites such as the gastrointestinal tract (particularly from the stools) must be interpreted with caution; repeated isolation is the most important criterion for assessing clinical significance.

## Identification of Mycobacteria

The very high prevalence of the species *Mycobacterium avium* among mycobacteria isolated from stools or intestinal tissues in AIDS does not mean that one may safely avoid identification. Other nontuberculous mycobacteria, whose pathogenicity is questionable, may rarely be isolated in such specimens, and *Mycobacterium tuberculosis* too may exceptionally be present.

The identification, which may require up to 8 weeks by conventional procedures, can nowadays be performed rapidly using genomic investigations or chromatographic analysis of cell wall mycolic acids.

### *Conventional Procedures*

Conventional procedures include cultural and biochemical tests. Cultural tests on solid media are able to distinguish slow from rapid growers, to determine the temperature range that supports growth of the strain, and to discern colony morphology and pigmentation. The latter features allow one to distinguish nonpigmented, nonchromogenic mycobacteria, from those that are pigmented, scoto- or photochromogenic (the latter developing pigment only with light exposure).

Special cultural tests are those in which media are supplemented with different additives. These tests allow differentiation of the organisms on the basis of the substances by which they are inhibited and of those that are tolerated. The best known such medium is Lowenstein-Jensen supplemented with 5% NaCl. A simple medium, such as MacConkey agar without crystal violet, may be used to differentiate less fastidious species that are able to grow on it.

Biochemical tests [14] are useful in differentiating various metabolic activities of the different strains.

The most widely used of these tests are those involving nitrate reductase; catalase, both qualitative (resistant or not at 68°C) and quantitative (producing a foam column higher or lower than 45 mm); urease; arylsulfatase; esterase (ability to hydrolyze Tween 80); tellurite reductase; and pyrazinamidase.

Species identification, once a number of conventional test results are available, is made with the use of tables (Table 1) provided by major microbiology textbooks. The lower the number of tests used, the higher the probability that the variability of even a single feature results in misidentification. In addition, conventional tests are not suitable for identification of rare or new mycobacterial species [15].

### Genomic Investigations

Genomic technologies which allow species identification include DNA-probe hybridization, genetic sequencing, and restriction fragment length polymorphism (RFLP) analyses.

#### *DNA Probes*

DNA probes are commercially available (AccuProbe, Gen-Probe, San Diego, Calif., USA; INNO LiPA Mycobacteriua, Innogenetics, Ghent, Belgium) for the most frequently encountered mycobacteria. They are based on the ability of the single-stranded DNA, forming the probe, to align and associate with a complementary, species-specific, tract of the bacterial genome to form a stable double-stranded complex.

In the AccuProbe, which is available for *Mycobacterium tuberculosis* complex, *Mycobacterium avium* complex, *Mycobacterium kansasii*, and *Mycobacterium gordonae*, the probe is complementary to a short sequence within the 16S rRNA and is labeled with chemiluminescent molecules (acridinium esters). While in hybridized probes the acridinium label is protected by the double-strand structure, in nonhybridized ones it remains exposed; the adjunct of hydrolyzing agent degrades acridinium ester in nonassociated probes so that the DNA-RNA hybrids may be disclosed by the chemiluminescence which develops after the addition of hydrogen peroxide. The species specificity of the probe therefore allows the identification of the strain.

AccuProbe originally intended for mycobacteria grown on solid media can also be used with strains developed in broth, once they are concentrated by centrifugation at 11,000×*g* for at least 15 min [16]. A loopful of cells from a colony grown on solid medium, or from the pellet obtained centrifuging a liquid culture, are mixed with 100 µl of lysis reagent and 100 ml of hybridization buffer in a tube containing glass beads. Once vortexed, the tube is sonicated for 15 min in order to lyse the cells and release the RNA, and after a 10-min inactivation at 95°C, 100 µl of the lysed specimen is transferred to the tube containing the lyophilized probe and is incubated at 60°C for 15 min to allow the hybridization. After the addition of 300 µl of selection reagent, which selectively hydrolyzes the chemiluminescent molecules of nonhybridized probes, the vortexed tube is incubated again at 60°C for 5 min and, once cooled, placed in the luminometer where the injection of hydrogen peroxide causes the development of chemiluminescence from the hybridized probe. The luminescence is measured and scored in relative light units (RLU). (The breakpoint has been fixed at 30,000 RLUs.)

The INNO LiPA assay, which is based on the principle of reverse hybridization, can simultaneously identify eight taxa and several intra-specific variants. Its 14 probes are complementary to a species-specific sequence of the DNA region spacing the 16S and 23S ribosomal RNA genes, and are immobilized as parallel lines on a membrane strip. The hybridization with the previously amplified target is detected by the development of a colored precipitate on the specific line-probe(s) following the addition of a conjugate enzyme and a chromogenic substrate.

After the extraction of bacterial DNA, obtained by boiling a bacterial suspension from a solid medium for 10 min, or by inactivating the pellet of a liquid medium by submitting it to 95°C for 30 min and then freezing it at –20°C for 30 min, the supernatant is used for polymerase chain reaction (PCR) amplification with biotinylated primers. The amplification product, after a 5-min contact with a denaturation solution at room temperature and the addition of a typing strip, is incubated in a 62°C shaking water bath for 30 min. After washing the strip and a 30-min incubation at room temperature with the added conjugate (streptavidin-alkaline phosphatase), the color development is carried out (after a further washing) by incubating the strip with the phosphorylated substrate buffer for 30 min. The identity of the reacting probes is indicated by the position of the colored bands. The detectable taxa include the *Mycobacterium tuberculosis* complex, *Mycobacterium kansasii*, *Mycobacterium xenopi*, *Mycobacterium gordonae*, *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium scrofulaceum*, and *Mycobacterium chelonae*. Within the species *Mycobacterium kansasii* and *Mycobacterium chelonae* intra-specific groups are distinguished. A presumptive identification of MAI-X group, and of the species *Mycobacterium malmoense* and *Mycobacterium haemophilum* as well, may be inferred by the hybridization with a poly-specific probe. A genus-specific probe reacting with all the species of the genus *Mycobacterium* is also available.

**Table 1.** Biochemical and cultural features of mycobacteria encountered in clinical specimens (modified from [69] with

| Descriptive term | Species | Optional growth temperature (°C) | Usual colony morphology | Pigmentation | Niacin | Grouth on TCH | Nitrate | Semi-quantitative catalase |
|---|---|---|---|---|---|---|---|---|
| Slow growers | | | | | | | | |
| TB complex | *M. tuberculosis* | 37 | R | N (100) | + (95) | + | + (97) | <45 (89) |
| | *M. africanum* | 37 | R | N | – | V | – | <45 |
| | *M. bovis* | 37 | R | N (100) | – (4) | – | – (9) | <45 (69) |
| | *M. bovis* BCG | 37 | R | N | – | – | – | <45 |
| Nonchromogens | MAC | 37 | S/R | N (87) | – (0) | + | – (4) | <45 (98) |
| | *M. haemophilum* | 30 | R | N | | + | – | <45 |
| | *M. malmoense* | 37 | S | N (88) | – (0) | + | – (1) | <45 (99) |
| | *M. shimoidei* | 37 | R | N | – | + | – | <45 |
| | *M. genavense* | 37 | St | N | – | + | – | >45 |
| | *M. celatum* | 37 | S/S | N (100) | – | + | – (0) | <45 (100) |
| | *M. ulcerans* | 30 | R | N | – | + | – | <45 |
| | *M. terrae* complex | 37 | SR | N (93) | – (1) | + | ± (67) | >45 (93) |
| | *M. triviale* | 37 | R | N (100) | – (0) | + | + (89) | >45 (100) |
| | *M. gastri* | 30 | S/SR/R | N (100) | – (0) | + | – (0) | <45 (100) |
| Chromogens | *M. kansasii* | 37 | SR/S | P (96) | – (4) | + | + (99) | >45 (93) |
| | *M. marinum* | 30 | S/SR | P (100) | ± (21) | + | – (0) | <45 |
| | *M. simiae* | 37 | S | P (90) | ± (63) | + | – (28) | >45 (93) |
| | *M. asiaticum* | 37 | S | P (86) | – (0) | + | – (5) | >45 (95) |
| | *M. xenopi* | 42 | S | S | – | + | – | <45 |
| | *M. gordonae* | 37 | S | S (99) | – (0) | + | – (1) | >45 (90) |
| | *M. scrofulaceum* | 37. | S | S (97) | – (0) | + | – (5) | >45 (84) |
| | *M. szulgai* | 37 | S/R | S/P (93) | – (0) | + | + (100) | >45 (98) |
| | *M. flavescens* | 37 | S | S (100) | – (0) | + | + (92) | >45 (94) |
| Rapid growers | | | | | | | | |
| Nonchromogens | *M. fortuitum* group | 28 | R/S | N (100) | – | + | + (100) | >45 (93) |
| | *M. chelonae* | 28 | S/R | N (100) | –/+ | + | – (1) | >45 (92) |
| | *M. abscessus* | 28 | S/R | N | – | | – | >45 |
| | *M. mucogenicum* | 28 | S | N | – | | V | >45 |
| | *M. smegmatis* | 28 | S/R | S (50) | – | + | + (95) | <45 (82) |
| Chromogens | *M. phlei* | 28 | R | S | – | + | + | >45 |
| | *M. vaccae* | 28 | S | S | – | + | + | >45 |

R, rough; S, smooth; N, nonchromogen; S, scotochromogen; P, photochromogen; V, variable; t, thin or transparent; SR,

### *Nucleotide Sequencing*

Genetic sequencing is still a technique out of reach of the majority of clinical laboratories; it is in fact generally performed with the aid of expensive automatic sequencers. The target of sequencing is represented by a genomic region in which highly conserved, constant nucleic acid stretches and variable ones coexist. The region most frequently aimed for this purpose is 16S ribosomal RNA or DNA [17] which is characterized by two hypervariable segments with species-specific nucleotide sequences (Fig. 9).

PCR carried out in parallel in four separate reaction environments each containing (along with the primers delimiting the target region and the usual amplification mixture) a different dideoxy dye terminator, produces amplicons of every possible length. Once such amplicons are sorted with electrophoresis on the basis of their length, the sequence of the target region is reflected by the terminal nucleotides of the ordered amplification products.

### *Restriction Fragment Length Polymorphism*

Restriction fragment length polymorphism (RFLP) allows one to cut DNA into fragments by using opportunely selected restriction enzymes. Once submitted to gel electrophoresis, the fragments produce polymorphic patterns that differ according to the length and number of the fragments [18]. Choosing as target the gene encoding for heat shock proteins, species-specific RFLP patterns are obtained which

permission)

| 68°C catalase | Tween hydrolysis | Tellurite reduction | NaCl tolerance | Iron uptake | Aryl-sulfatase 3 day | Mac Conkey | Urease | Pyrazina-midase 4 day |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | ± (64) | |
| – (1) | ± (68) | ± (36) | – (0) | – | – (0) | – | | + |
| – | – | – | – | – | – | – | | – |
| – (2) | – (21) | | – (0) | – | – (0) | – | + | – |
| – | ± | | – | – | – | | ± (50) | – |
| ± (60) | – (2) | + (81) | – (0) | – | – (1) | ± | + | + |
| – | – | – | – | – | – | – | – (0) | + |
| ± | + (99) | + (74) | – (0) | – | – (0) | | – | + |
| – | + | | – | – | – | – | – (9) | + |
| + | + | | | – | – | | – | + |
| + (100) | – (0) | + (100) | – (0) | – (0) | + (100) | – (0) | + | + (100) |
| + | – | | – | | – | | – (0) | – |
| + (92) | + (99) | ± (46) | – (2) | – | – (2) | V | V | V |
| + (100) | – (100) | – (25) | + (100) | – | ± (56) | – | – (13) | V |
| – (11) | + (100) | ± (50) | – (0) | – | – (0) | | ± (33). | – |
| + (91) | + (99) | ± (31) | – (0) | – | – (0) | – | ± (44) | – |
| – (30) | + (97) | ± (39) | – (0) | – | ± (41) | – | ± (49) | + |
| + (95) | – (9) | + (82) | – (0) | – | – (0) | | + (83) | + |
| + (95) | + (95) | – (20) | – (0) | – | – (0) | | ± (69) | – |
| ± | – | | – | – | + | | – (10) | |
| + (96) | + (100) | – (29) | – (0) | – | V | – | – | ± |
| + (94) | – (2) | ± (64) | – (0) | – | V | – | V (31) | ± |
| + (93) | ± (49) | ± (53) | – 0 | – | V | – | V (31) | + |
| + (100) | + (100) | ± (44) | ± (62) | – | – (0) | – | + (72) | + |
| | | | | | | | + (72) | |
| + (90) | ± (43) | + (92) | + (85) | + | + (97) | + | | + |
| ± (53) | ± (39) | + (89) | V | – | + (95) | + | + (70) | + |
| | V | | ± | – | + | + | + (89) | |
| | + | | – | | + | + | + | |
| – | + | + | + | + (100) | – (0) | + (100) | + | |
| + | + | + | + | + | – | – | | |
| + | + | + | V | + | – | – | | |

intermediate in roughness.

allow the identification of the strain [19]. Not infrequently, different patterns will appear within members of a single species.

### *Chromatographic Analysis of Cell Wall Mycolic Acids*

Mycolic acids are branched long-chain fatty acids present in the cell wall of mycobacteria. Seven types of mycolic acids, differing in length of the chain and in the presence of functional groups, are known, which are differently combined in the various species of the genus *Mycobacterium* [20, 21]. Various chromatographic techniques can be used to sort mycolic acids, or their fragments, and consequently to distinguish species. The extraction of mycolic acids from bacterial colonies is the preliminary step in any technique chosen.

#### *Thin-Layer Chromatography*

Thin-layer chromatography uses silica plates (stationary phase) on whose surface the mycolic acids in the sample are separated as result of the advance of a solvent (mobile phase). Once stained, they appear as spots and are identifiable by their positions as compared with spots made by known mycobacterial species [22, 23]. Most species, which have no more than two or three different mycolic acids, are characterized by species-specific patterns (Fig. 10); but not infrequently two or more species share a pattern.

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129                          172
TGA TCT GCC CTG CAC TTC / TAC CGG ATA GG-ACCA CGG GAT GCA TGT CT-TGT GGT GGA     M. tuberculosis
.A. ... ... ... ... ...   ... ... ... ..-...T ..A .GC ... ..C ..-... ... ...   M. interjectum
.A. ... ... ... ... ...   ... ... ... ..-.... .TT .GC ... ..G ..-... ... ...   M. simiae
... ... ... ... ... ..T   ... ..A ... TTCC.TA TT. .TC ... ..G .CTG.. A.G ...   M. flavescens
... ... ... ... ... ..T   ... ..A ... ..-...G .AT .C. ... ..G TG-... ... ...   M. nonchromogenicum
... ... ... ... ... .CT   ... ... ... ..-.... T.. ... ... ... TC-... ... ...   M. terrae
... C.. ... ... ... ...   ... ... ... ..-.... TTC TGC ... ... GG-G.. ... ...   M. xenopi
.A. ... ... ... ... A..   ... ..A ... ..-.... .A. ..C A.. ... .C-... ... ...   M. gordonae
C.. ... ... ... ... ...   ... ... ... ..-.... ... ... T.. ... .C-... ... ...   M. marinum/M. ulcerans
CA. ... ... ... ... ...   ... ... ... ..-.... .TT .GC ... ..C ..-... ... ...   M. scrofulaceum
.A. ... ... ... ... ...   ... ... ... ..-...C ..A .GC ... ..C ..-..G ... ...   M. szulgai
.A. ... ... ... ... ...   ... ..A ... ..-...C ..A .GC ... ..C ..-..G ... ...   M. malmoense
CA. ... ... ... ... AC.   ... ... ... ..-.... .TT .GC ... ..C. .-... ... ...   M. gastri/M. kansasii
CA. ... A.. ... ... ...   ... ... ... ..-...T .AA ..C ... .... .-.C. ... ...   M. avium
CA. ... ... ... ... ...   ... ... ... ..-...T TTA .GC ... .... .-.TA ... ...   M. intracellulare
.A. ... ... ... ... ACT   ... ... ... ..-...T .TC .GC ... ..C. .AG.A ... ...   M. intermedium
.A. ... ... ... ... ...   ... ... ... T.-.... ... A.C ... ...T .-... ... ...   M. genavense
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**Fig. 9.** Sequences of hypervariable region A within the 16S rRNA of several common mycobacterial species. Positions are indicated by *Escherichia coli* alignment, only nucleotides different from *M. tuberculosis* are shown; dashes indicate deletions

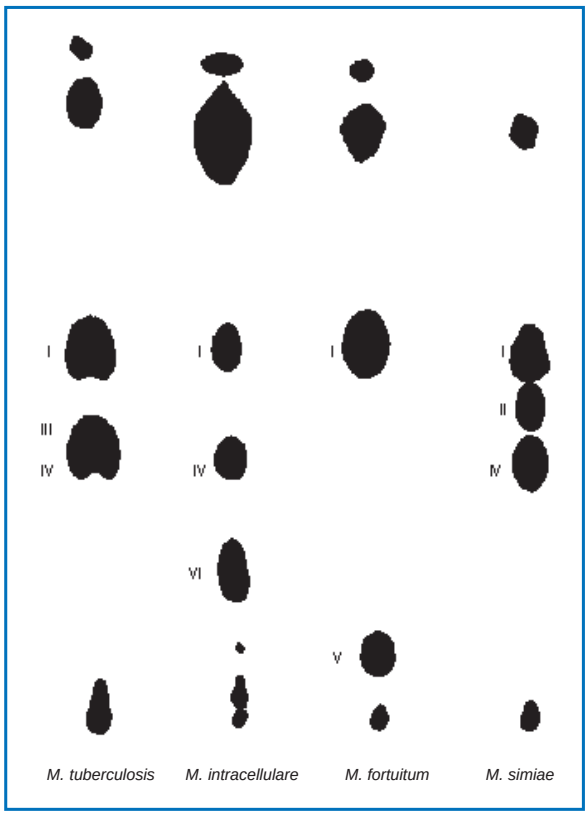


**Fig. 10.** Thin-layer chromatography patterns of four mycobacterial species. *M. tuberculosis*; *M. intracellulare*; *M. fortuitum*; *M. simiae*. I, alpha-mycolates; II, alpha'-mycolates; III, methoxy-mycolates; IV, keto-mycolates; V, epoxy-mycolates; VI, wax-ester-mycolates. (From [67] with permission)

*Gas-Liquid Chromatography*

Gas-liquid chromatographic analysis uses a gas (mobile phase) and a liquid (stationary phase) to separate the short chain fatty acids and the fragments of mycolic acids obtained by means of thermal cleavage of the latter (Fig. 11) [24]. The recognition of these fragments is usually obtained by mass spectrometry [25]. Cleavage products are consistent within single mycolic acids and consequently their pattern is constant within a species. A software program is commercially available that is suitable for identification of a vast variety of bacteria, among which are about 30 mycobacterial species [26]. The major problem with this procedure is the high number of species that share a common gas-chromatographic pattern.

*High-Performance Liquid Chromatography*

High-performance liquid chromatography (HPLC) uses high pressure to carry the liquid mobile phase, containing the sample, through the solid stationary phase present in the column. The various fractions of mycolic acids, previously saponified, extracted and derivatized to bromophenacyl esters, are separated in the column and eluted at different times (retention times) [27–31]. The detector, on the basis of individual ultraviolet absorbance, plots single fractions as picks arranged in a profile that is sufficiently different from those of other species so as to provide identification when compared with profiles of known mycobacteria (Fig. 12). A commercially available software package interprets profiles and supplies affordable identifications of the most frequently

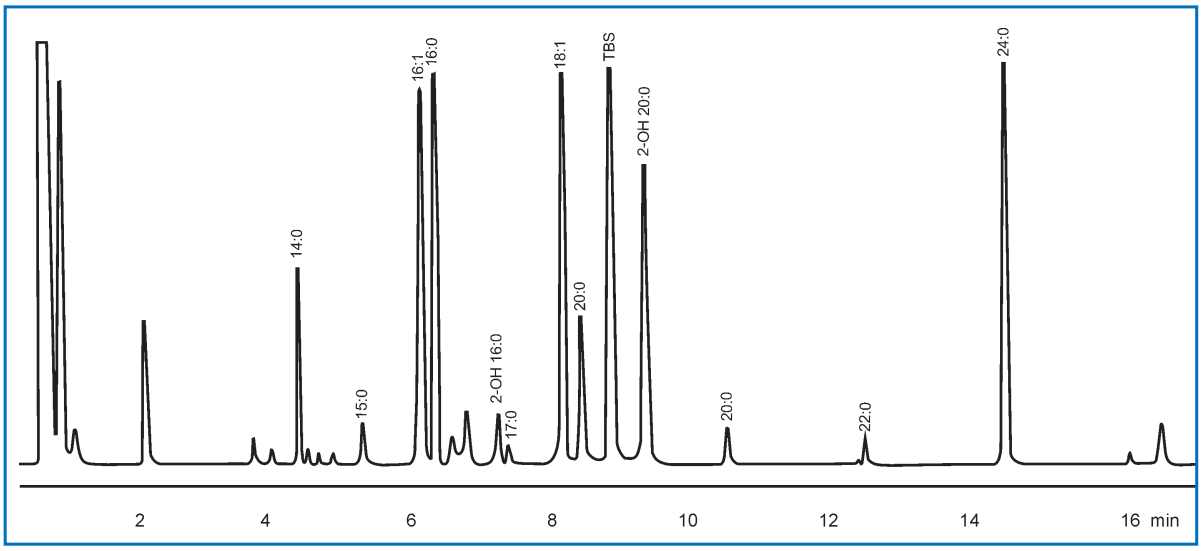


**Fig. 11.** Gas-chromatographic pattern of *Mycobacterium avium-intracellulare*. (From [67] with permission)

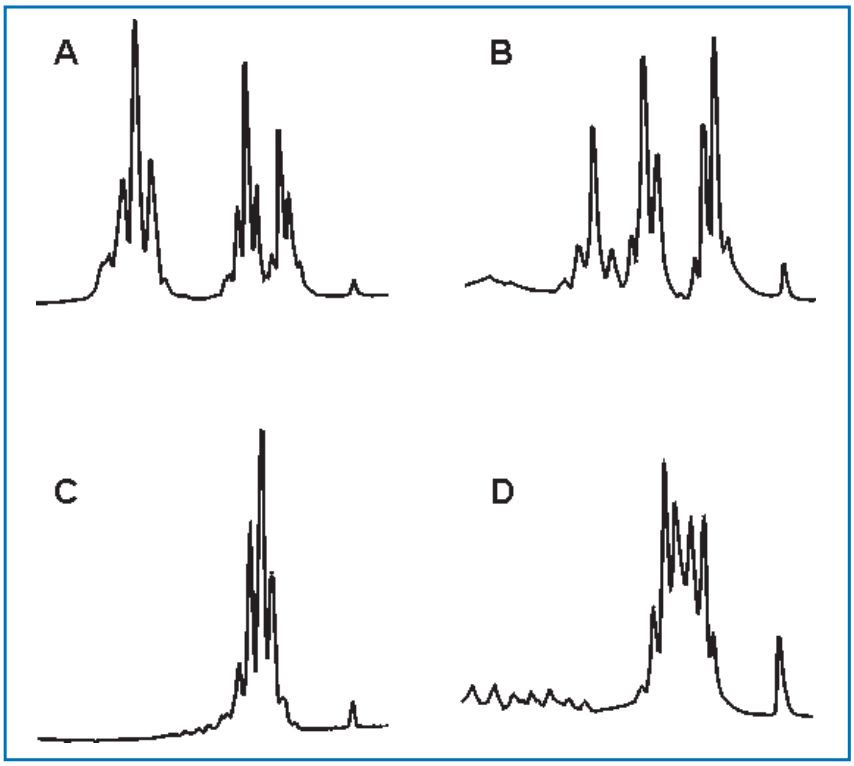


**Fig. 12.** High performance liquid chromatography of: *M. intracellulare*; *M. simiae*; *M. tuberculosis*; *M. kansasii*

detected mycobacterial species and may represent a useful help for unskilled microbiologists [32].

A fluorescence-based detection system, more sensitive than the ultraviolet-based system, may also be used to detect HPLC products.

Still under investigation is the possible use of HPLC for identification of mycobacteria at an early stage of growth in liquid culture [33].

## Amplification Techniques

Culture-free techniques, based on direct amplification and detection of selected species-specific genomic segments, have been developed in recent years. The most frequently used procedure is the PCR, which produces exponential doubling of the target DNA. Such amplification can potentially detect

**Table 2.** Concentrations of drugs used for radiometric susceptibility testing of *Mycobacterium avium* complex

| Antibiotic | Concentration (μg/ml) | Diluent |
|---|---|---|
| Amikacin | 8–4–2 | Distilled water |
| Ciprofloxacin | 4–2–1 | Distilled water |
| Clarithromycin | 32–8–2 | Methanol/distilled water |
| Clofazimine | 0.25–0.12–0.06 | Dimethyl-sulfoxide |
| Ethambutol | 8–4–2 | Distilled water |
| Rifabutin | 2–0.5–0.12 | Methanol/distilled water |
| Rifampicin | 8–2–0.5 | Methanol/distilled water |
| Streptomycin | 8–4–2 | Distilled water |

very small numbers of bacterial cells, a sensitivity even greater than that of culture; it is, however, unlikely to replace culture of mycobacteria. Only culture allows one to monitor the response to therapy and to test antimicrobial susceptibility.

One of the genomic zones most frequently chosen as an amplification target is the gene encoding for the ribosomal 16S RNA, which is characterized by the presence of species-specific sequences.

PCR is widely used for direct detection of *Mycobacterium tuberculosis*, but some work has also been done in setting up a procedure suitable for direct detection of other species. Apart from a number of "in house" techniques used mainly for research, a PCR technique for detection of *Mycobacterium avium* complex (MAC) and a genus-specific one for the amplification of every organism belonging to the genus *Mycobacterium* are commercially available today in a fully automated version (Amplicor, Roche, Branchburg, NJ, USA) [34, 35].

## Susceptibility Testing

The proportion method developed for *Mycobacterium tuberculosis* is based on a 1% resistance criterion: that is, a strain is considered resistant to a drug when the portion of resistant mutants is 1% or greater [36]. The proportion of such mutants varies in wild strains (i.e., the strains isolated from patients never treated with that particular drug) from $10^{-7}$ to $10^{-11}$ depending on the antimicrobial tested.

No reference method exists at present for susceptibility testing of nontuberculous mycobacteria. The proportion method cannot be used for nontuberculous species as, in contrast to *Mycobacterium tuberculosis*, they are characterized by a wide range of minimal inhibitory concentrations (MIC) that makes the choice of the critical concentration problematic. Furthermore, isoniazid and pyrazinamide, among first-line antituberculous drugs, are not active against nontuberculous mycobacteria which, in contrast, may be variably susceptible to macrolides, quinolones, clofazimine, rifabutin, and amikacin.

Only for MAC, which includes the most frequently encountered nontuberculous species, have several techniques been proposed for testing antimicrobial susceptibility. Because of the difficulties of determining unequivocally critical concentrations, which are necessary for developing a qualitative method, labor-intensive quantitative dilution tests are required. These tests, based on MIC determination, have been developed both as a micromethod and a macromethod.

Among the latter, an interesting rapid technique has been developed that employs the radiometric Bactec system; it uses three concentrations of each drug and allows determination of MICs in 5–8 days [37] (Fig. 13). For each drug the lowest concentration has been set equal to the critical concentration adopted for *Mycobacterium tuberculosis* susceptibility testing, and represents the cutoff between full and moderate susceptibility, while the highest concentration corresponds to the maximum concentration attainable in vivo and represents the cutoff between moderate and full resistance. Finally, an intermediate concentration distinguishes moderate susceptibility from moderate resistance (Table 2). Two controls are used, the lower a 100-fold dilution of the other, and strict rules allow one to recognize errors due to low or excessive inoculation. The undiluted control must not exceed the maximum of the Bactec scale in the first 3 days, while the diluted control has to reach a value of growth index (GI) greater than 20 no later than the sixth day, and then not to decrease in the next 2 days. Finally, a strain is considered susceptible to the lower drug concentration in presence of which the daily GI never exceeded 50 and the daily increase in GI was never higher than that of the diluted control.

Minimal bactericidal concentration (MBC) can also provide useful information, being the lowest drug concentration not allowing any growth in a subculture on solid medium, determined by seeding a drop from every broth with a drug concentration equal or greater than the MIC on such a solid medium.

Since MAC is usually resistant to most drugs, the assessment of possible synergism among drugs is important. Two drugs are considered synergic if they are active in combination at concentrations equal to at least a fourfold decrease of the MICs. This is unfortunately a time-consuming and expensive determination.

Because agreement of in vitro data with in vivo response has been found questionable, standardized protocols are recommended at present for the treat-

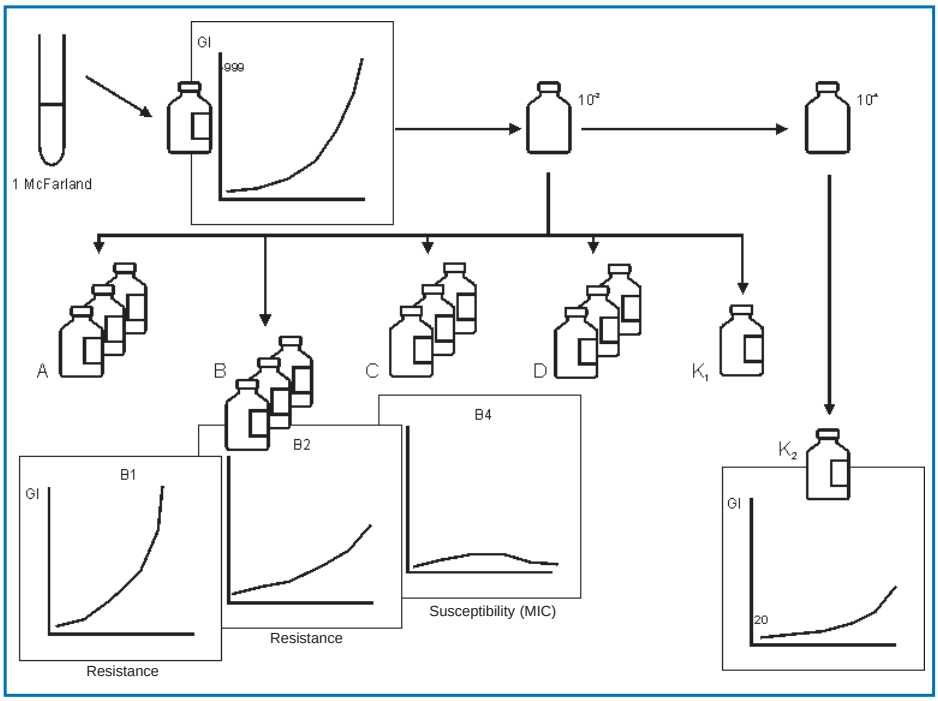


**Fig. 13.** Radiometric determination of minimal inhibitory concentrations of *Mycobacterium avium* complex

ment of *Mycobacterium avium* infections, with results of susceptibility testing being ignored. A further ground of doubt is the disagreement between MIC results obtained on solid vs. liquid media, the latter giving consistently lower results, considered the more reliable. Susceptibility testing is even more unreliable for nontuberculous mycobacteria other than MAC.

The most frequently used therapies at present combine at least three agents including ethambutol, a macrolide, and a rifamycin or a quinolone. Single drugs (rifabutin or clarithromycin) are used only for prophylaxis in patients with $CD4^+$ counts less than $100/mm^3$.

### Serotyping

Historically, serotyping of MAC has been performed by means of a seroagglutination reaction whose target is an oligosaccharidic polymorphic antigen present in the C-mycoside of the cell wall. Using specific antisera it is possible to split the MAC into 28 different serovars (serotypes), ten of which belong to the species *Mycobacterium avium* and ten to the species *Mycobacterium intracellulare*, with no consensus on assigning the eight remaining serovars [38]. At present, seroagglutination is only exceptionally used; typing is preferably done using monoclonal antibodies with the ELISA technique [39], or by identifying previously extracted oligosaccharidic antigens by means of thin-layer chromatography [40].

### Molecular Epidemiology

The epidemiology of MAC at a molecular level has recently been studied. The target of such investigations has most often been a motile element (insertion sequence) that may be found in a variable number of copies in the chromosome. The most frequently investigated is IS*1245*, of which three copies have been found in the strains isolated from birds and about 20 in those from humans and pigs [41]. The use of enzymes (endonucleases) specific for a restriction site present only once within IS*1245* (restriction sites outside the insertion element are unimportant) allows one to cut the chromosome into at least as many fragments as there are insertion elements present in the strain. The fragments, electrophoretically separated, (Fig. 14), are recognized by using the Southern blot technique, and by hybridizing a sequence of the insertion element with a complementary probe. In addition to their number, the molecular weight of restriction fragments also differentiates strains. Such differentiation makes it possible to track the epidemiological route of MAC infections at the molecular level.

## Microbiology

### *Mycobacterium avium* Complex

The *Mycobacterium avium* complex (MAC) includes the species *Mycobacterium avium* and *Mycobacteri-*

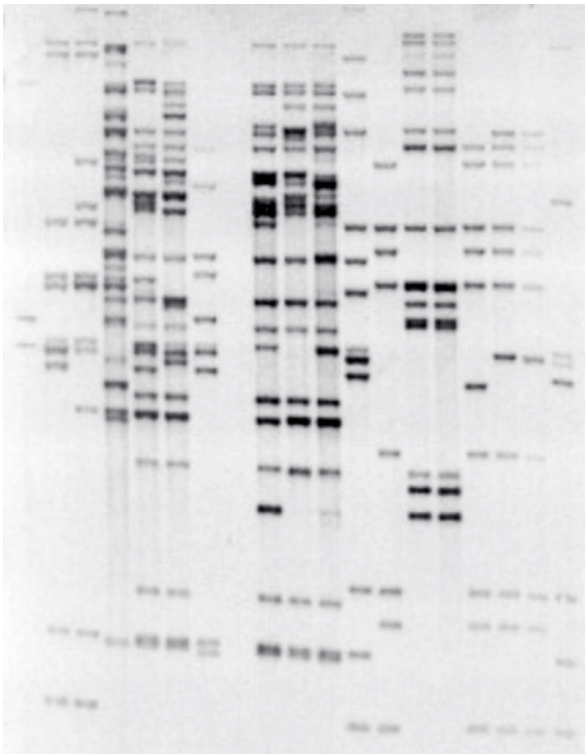

**Fig. 14.** Restriction fragment length polymorphism patterns of several strains of *Mycobacterium avium*. (From [68] with permission)

*um intracellulare*, which are undistinguishable with conventional biochemical and cultural tests, and a further group of related mycobacteria (MAI-X group) not yet assigned to any species [42]. The recognition of members of MAC is, however, possible by using commercial DNA probes: *Mycobacterium avium* and *Mycobacterium intracellulare* each hybridize with a specific probe, and both with the probe recognizing the whole complex. MAI-X organisms in contrast hybridize with the latter probe only.

The microscopic morphology of MAC is variable. Along with the predominant coccobacillar form, we sometimes find filamentous morphology and, even less frequently, a branched form.

Traditionally, MAC is classified within the group of nonpigmented, slow-growing acid-fast bacilli; this taxonomic position may still be considered valid, at least for didactic purposes, despite the rapid growth of several strains in liquid medium and the yellow pigmentation presented by some of them with aging.

With conventional tests, MAC appears biochemically inert, being characterized by a battery of negative reactions.

An interesting feature of MAC is the occurrence of three colony variants: the opaque type (Fig. 5), which is smooth and domed; the transparent type (Fig. 15), which is smooth and flat; and the rough type. Rough colonies are rare and are not found in primary isolations, but are probably generated by the loss of cell-wall glycopeptidolipids [43]. The two principal forms can be found together in vitro, and the transparent variant tends to covert into the opaque form while the reverse happens much less frequently.

In AIDS patients with disseminated MAC disease, however, the transparent type is isolated almost exclusively. Important features are linked to the two major colonial phenotypes: the transparent variant is in fact more virulent [44] than the opaque variety and consistently more resistant to antimicrobial agents [45].

MAC is ubiquitous in the environment and can be isolated from water, soil, plants, animals, and house dust [46]. The direct transmission of MAC from animals to humans is probably very rare and even lower is the risk of human-to-human transmission.

Members of MAC are intrinsically resistant to almost all antimycobacterial drugs, probably because of the impermeability of their cell wall [45]. A confirmation of this hypothesis seems to come from the apparent enhancement of antimycobacterial agents (almost all of them) brought about by surfactant agents such as Tween 80 [47], which alters the cell wall architecture, and from the synergistic effect with ethambutol, whose target is the cell wall [48]. The nature of the colony-type-related antimicrobial susceptibility changes of the MAC is, on the contrary, adaptive and may be variously expressed.

Human diseases caused by MAC have been documented for many years [49] but it is with the emergence of the AIDS epidemic that their prevalence significantly increased.

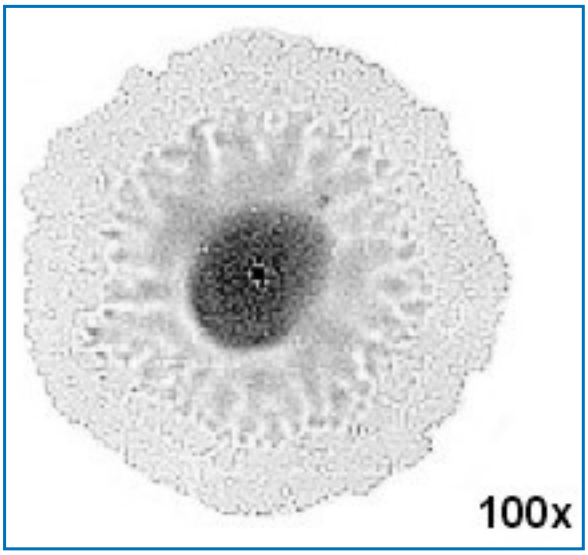


**Fig. 15.** *Mycobacterium avium* complex, transparent type microcolonies

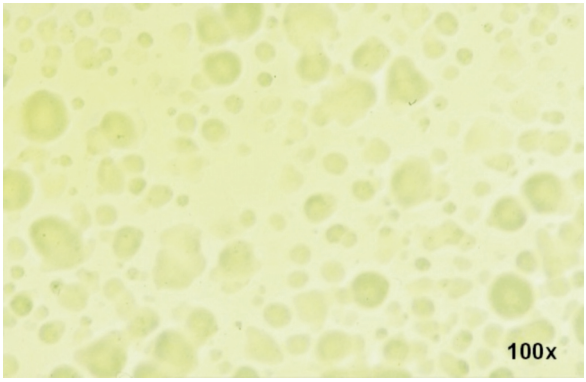


**Fig. 16.** *Mycobacterium genavense*, microcolonies

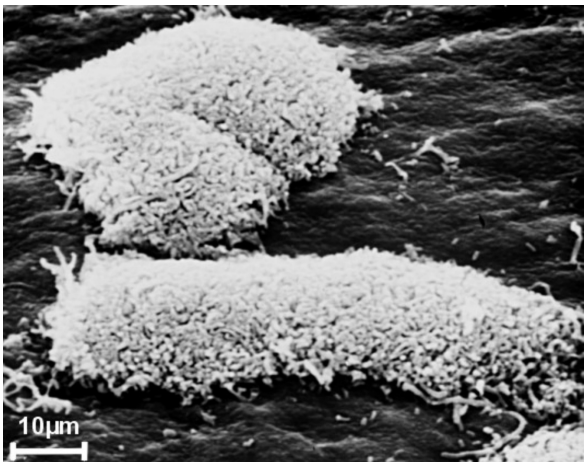


**Fig. 17.** *Mycobacterium genavense*, microcolonies (electron microscopy). (From [13] with permission)

In non-AIDS patients, the disease almost exclusively affects the respiratory apparatus, mainly in elderly people with predisposing conditions. Cavitary infiltrates are the most frequent manifestation. MAC is moreover the most frequent agent of cervical lymphadenitis, not rare in children 1–5 year old [50]. In immunodeficiency virus-infected patients, disease involving single organs and generalized infections are both frequent.

Progressive immunodeficiency is the most important risk factor for MAC disease in AIDS patients and may be said to represent the natural outcome for those who survive other opportunistic infections. It is in fact about ten times more frequent in subjects with $CD4^+$ lymphocyte counts of less than 10/mm$^3$ in comparison with patients with counts of more than 100/mm$^3$ [51]. MAC infection has been detected at autopsy in about 50% of AIDS patients [52].

The most important portals of entry are the intestinal mucosa and, less frequently, the bronchi. The first step in disseminated MAC infection is generally the colonization of the intestinal tract, which is often followed by infiltration of the mucosa and, a few months later, by bacteremia. Gut disease is characterized by intestinal erosion due to penetration of bacteria into the lamina propria and by massive involvement of mesenteric lymph nodes and Peyer's patches. The most frequent symptom is chronic diarrhea. Duodenal or rectal biopsies are often diagnostic. That mycobacteria are very frequently isolated from stools, this may reflect both a colonization and an infection and represent an important risk factor for dissemination of disease.

Members of MAC survive and multiply within phagocytes also, thanks to their ability to inhibit the fusion of phagosomes with lysosomes and thus prevent acidification of the intracellular environment [53].

In patients with AIDS, the species *Mycobacterium avium* is the almost exclusive cause of MAC disease while in non-HIV-infected subjects it is involved in no more than 60%, the remainder being due to *Mycobacterium intracellulare* [54].

Several studies seem to suggest that particular MAC types infecting AIDS patients possess specific genetic determinants that confer the ability to penetrate and multiply within macrophages, thus contributing to the worsening of immunodeficiency [55].

Despite all that has been said about the prevalence and seriousness of MAC infection in immunosuppressed patients, the number of cases in which MAC infection is the AIDS-defining disease is still scant.

The introduction of highly effective retroviral-protease inhibitors appeared at one time to have almost completely eliminated the nontuberculous mycobacterioses in AIDS patients. More recently it has become evident that, although with different features, the problem still exists. While in previous years the progressive decrease of $CD4^+$ lymphocytes was responsible for the dissemination of MAC infection, we have now come to see, with restoration of a more efficient immune defense system, the emergence of the so-called immuno-reconstitution syndrome. In subjects with this condition, MAC organisms colonizing the patient are inhibited from dissemination by the revitalized immune system, but localized granulomatous lesions, generally limited to lymph nodes, have been seen increasingly.

## *Mycobacterium genavense*

An unidentified mycobacterium repeatedly isolated from AIDS patients since the late 1980s [56] has been recognized in 1992 as belonging to a new species

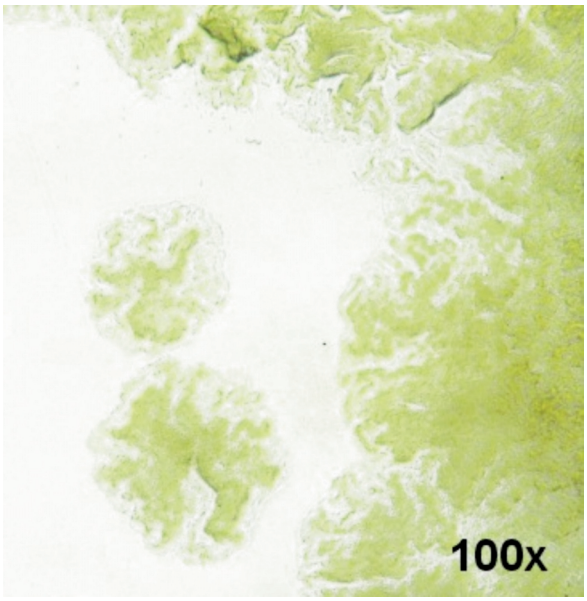


**Fig. 18.** *Mycobacterium kansasii*, microcolonies

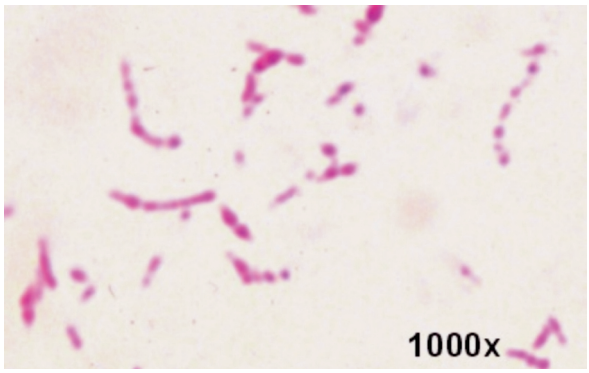


**Fig. 19.** *Mycobacterium kansasii*, cross-banded bacilli

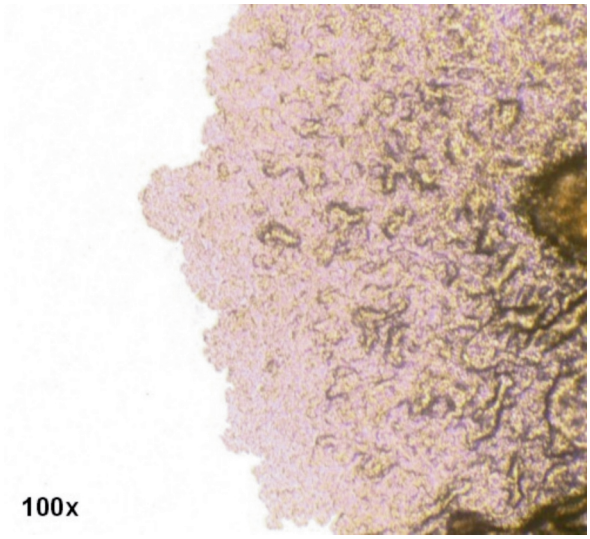


**Fig. 20.** *Mycobacterium tuberculosis*, microcolony

named *Mycobacterium genavense* [57]. The main reason for the delayed recognition of this novel mycobacterium is its inability to grow on conventional solid media; its growth is in fact extremely slow and only in liquid media. It is cultured mainly in radiometric Bactec, and among solid media only on Middlebrook agar enriched with mycobactine (a compound related to iron metabolism, produced by almost all mycobacterial species) (Figs. 16, 17) or with human blood. The first recognition of the strain was possible only thanks to molecular biology techniques: genetic sequencing of previously PRC-amplified mycobacterial 16S rDNA revealed a unique sequence [58]. Because of its fastidious nature, *Mycobacterium genavense* cannot be identified with conventional tests or any others that require a large biomass. In addition to molecular biology methods, lipidic techniques are able to identify the organism, mainly by using HPLC, which reveals a profile similar to, but nevertheless distinguishable from, that of *Mycobacterium simiae* [13].

Almost all isolations of *Mycobacterium genavense* from humans have been obtained from AIDS patients with $CD4^+$ cell counts of less than 50/µl, thus suggesting an epidemiology similar to the disseminated infections of MAC [59]. There have been many isolations of *Mycobacterium genavense* from birds [60], evoking the hypothesis that birds are a natural reservoir for this organism.

The disease and its symptoms suggest the gastrointestinal route of infection. The small intestines, lymph nodes, and spleen are the most frequently involved, the last two being enlarged and filled of histiocytes packed with acid-fast bacilli. Inflammatory reaction is minimal. The most common clinical manifestations are abdominal pain, chronic diarrhea, hepatosplenomegaly, lymphadenopathy, weight loss, and anemia [61]. Not infrequently the stools are heavily charged with acid-fast bacilli. Blood and bone marrow are perhaps the best sources of positive cultures of *Mycobacterium genavense*. However, even in liquid medium the growth is extremely slow, and up to 8 weeks may be necessary before obtaining the first feeble signal in radiometric broth; a less leisurely growth can be expected in slightly acidic media. The scant metabolic activity detectable with radiometric culture does not reflect, as for other mycobacterial species, a poor growth: smears prepared from Bactec vials showing a very low growth index may in fact contain many coccobacillary rods.

*Mycobacterium genavense* differs from other nontuberculous mycobacteria in its inhibition by *p*-nitro-acetyl-amino-β-hydroxypropiophenone (NAP). The handy NAP-test, used in the radiometric system to differentiate *Mycobacterium tuberculosis* from other mycobacterial species, is therefore suitable for preliminary identification [13]. Only molecular biology techniques, however, allow a definitive identification and, as many strains of *Mycobacterium genavense* fail to grow even in liquid media, these procedures also represent the method of choice for diagnosis.

Nothing is known about the antimicrobial susceptibility of *Mycobacterium genavense*. Treatments with drugs commonly used against disseminated MAC infection are, however, effective, and several procedures tentatively used to test susceptibility in vitro seem to suggest resistances less severe than those of MAC [13].

Almost 100 cases of disseminated infection with *Mycobacterium genavense* have been reported from different geographic locations. The real prevalence is no doubt much higher, and more than 10% of disseminated mycobacterial infections in AIDS are estimated to be due to *Mycobacterium genavense*. Few of these infections are diagnosed, not only because of the inconsistent growth of the organism, but also because they are commonly lost when present in mixed cultures [62].

### Rarely Encountered Mycobacterial Species

Intestinal infections due to other mycobacteria are rare in AIDS.

A few cases due to *Mycobacterium kansasii* have been reported [63]. In the scattering of HIV patients who are infected, a dissemination is not exceptional. In these patients, the bowel and mesenteric lymph nodes may be involved with caseating granulomas and an accumulation of foamy histiocytes.

*Mycobacterium kansasii* (Fig. 18) was one of the first nontuberculous mycobacteria described, and its detection in non-AIDS patients is not rare. The high isolation rates in several regions of the United States and in many central European countries are well documented [64]. A chronic pulmonary disease is the most common manifestation in nonimmunocompromised subjects, who often have predisposing pulmonary disorders.

The salient microbiological feature of *Mycobacterium kansasii* is its photochromogenicity. The organism in fact depends on light exposure for the production of carotene, which confers yellow pigmentation to the colonies.

Bacilli, which are long and broad, may present a characteristic cross-banding, particularly when cultured in liquid medium. (Fig. 19).

The species is usually susceptible to antimycobacterial drugs apart from isoniazid; a combination of drugs that includes rifampin is the cornerstone of the treatment of *Mycobacterium kansasii* disease [65].

The discovery by Robert Koch of tubercle bacillus as the cause of tuberculosis is one of the milestones in the history of microbiology. The disease may affect every part of the infected organism, and also the generalized form miliary tuberculosis is well known. The most frequent clinical manifestation remains pulmonary tuberculosis, a slowly progressive inflammatory process characterized by necrosis and caseation. When the process breaks into bronchi not only does disease spread to other areas of the lung, but many bacilli are coughed out that may infect others. Tuberculosis is a frequent complication in patients with AIDS, in which it is often characterized by lack of granuloma formation and by a tendency toward more rapid progression. Episodes of septicemia are not exceptional. Intestinal localization of disease, although rare, has also been reported [66].

*Mycobacterium tuberculosis* bacilli are thin, curved, and strongly acid-fast. The growth in culture is slow and colonies are typically rough (Fig. 20). When cultured on agar-based media, the microcolonies, observed microscopically, are readily recognized by presence of serpentine cord formations. Accumulation of niacin in the medium, nitrate reduction, and the lack of catalase activity after 20-min incubation at 68°C are the key identifying features that allow differentiation of *Mycobacterium tuberculosis* from all other mycobacterial species. Highly specific DNA probes (AccuProbe, INNO LiPA Micobacteria) are commercially available. They are, however, unable to distinguish *Mycobacterium tuberculosis* from other species included in *Mycobacterium tuberculosis* complex.

Although many other mycobacteria have been isolated in AIDS patients, their presence in intestinal or fecal samples is negligible.

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