Introduction and Evaluation of Novel Methods for the Diagnosis of Tuberculosis

PhD Thesis

by Dr. Ákos Somosközi

From the Department of Respiratory Medicine, Semmelweis University of Medicine, Budapest, Hungary

Supervisor: Dr. Judit Deák

Budapest 1999

Contents:

Summary:

The definitive diagnosis of tuberculosis is dependent on the isolation, identification and drug susceptibility testing of the causal agent *Mycobacterium tuberculosis* (*M. tuberculosis*) by cultivation. The past few years have brought significant changes to clinical mycobacteriology with the introduction of broth-based cultivation and molecular biological methods. However, the value of any new method can be estimated onlz on the basis of comparisons with conventional techniques currently available in clinical laboratories.

Microscopy is at present the fastest, cheapest and most easily performed technique in mycobacteriology. However, the sensitivity of microscopy is unsatisfactory and it is time-consuming. To eliminate these drawbacks, we have constructed a computer-directed automated microscope. Following evaluation of the equipment, we conclude that the automated microscope is able to detect acid-fast bacteria, the examination procedure with the instrument is more rapid and it is always possible to follow the standard recommendations of microscopy.

Cultivation on solid media, such as that of Löwenstein-Jensen (LJ), is both time-consuming, taking up to 6-8 weeks, and also insensitive. Broth-based cultivation can considerably shorten the time to detection of mycobacteria and increase the sensitivity of isolation. The rate of recovery and the mean time to detection of mycobacteria in clinical specimens were assesed with two new, non-radiometric broth-based systems, the Mycobacteria Growth Indicator Tube (MGIT) and MB Redox. These data were compared both with each other and with those obtained for the LJ and Middlebrook 7H11 reference media. We conclude that the MGIT and MB Redox systems are rapid, sensitive and easy to handle, and do not require additional costly instrumentation. In summary, both MGIT and MB Redox can be viable tools in the routine mycobacteriological laboratory.

Automation of the cultivation process is highly desired by laboratories dealing with a large number of specimens daily. The recently developed MB/BacT (Organon Teknika, Turnhout, Belgium) system is a fully automated, continuous monitoring system, which employs a colorimetric sensor and reflected light to monitor the production of CO_2 dissolved in the culture medium. In this study, we compared MB/BacT with the reference Bactec 460 TB system and LJ, and evaluated the performance of this new equipment in terms of sensitivity, time to detection of *M. tuberculosis*, handling requirements and degree of automation. In summary, the MB/BacT system has been shown to be a highly sensitive method for the culturing of clinical specimens for the recovery of *M. tuberculosis*. However, we conclude that MB/BacT requires further improvements as concerns the time to detection of *M. tuberculosis* and the capacity.

The goal of research in rapid diagnostics is the development of reliable procedures that can detect and identify mycobacteria directly in clinical specimens and avoid the many weeks otherwise required for isolation and identification by cultivation. The greatest breakthrough came from biotechnology with the application of nucleic acid amplification techniques. For any assay to be useful in a clinical diagnostic setting, a careful optimization of the amplification conditions and the standardization of protocols and reagents are needed to ensure proper performance. In the recently commercially developed Amplicor MTB polymerase chain reaction (PCR) assay (Roche Molecular Systems Inc., Branchburg, NJ, U.S.A.), reagents are standardized and quality-controlled to ensure performance. The purpose of our study was to compare the Amplicor PCR test for the direct detection of *M. tuberculosis* in respiratory specimens with conventional culture and staining techniques. We conclude that the first Hungarian study on application of PCR for the direct detection of *M.*

tuberculosis has demonstrated the high sensitivity and excellent specificity of Amplicor PCR.

Between 1996 and 1998 our laboratory introduced four broth-based cultivation methods in Hungary. We conclude that MGIT and MB Redox can also be viable alternatives to the Bactec 460 TB system. As the sensitivity and the rapidity of cultivation are significantly higher when liquid and solid media are used in combination, the wider introduction of liquid media in Hungary should be a must. The local validation of Amplicor PCR demonstrated that the majority of *M. tuberculosis*-positive specimens can be identified rapidly by means of the assay and, in consequence of the high negative predictive value, the Amplicor PCR test can help to exclude tuberculosis in 24 hours.

Abbreviations:

AFB	acid-fast bacteria
CFU	colony formation units
DNA	deoxyribonucleic acid
dUTP	2'-deoxyuridine-5'-triphosphate
GI	growth index
LJ	Löwenstein-Jensen
MGIT	Mycobacterium Growth Indicator Tube
M. tuberculosis	*Mycobacterium tuberculosis*
NPV	negative predictive value
NTM	non-tuberculous mycobacteria
PACT	polymyxin B, amphotericin B, carbenicillin, trimethoprim
PANTA	polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin
PCR	polymerase chain reaction
PPV	positive predictive value
rRNA	ribosomal ribonucleic acid
ZN	Ziehl-Neelsen

Preface:

This thesis is based on the following articles, referred in the text by their Roman numerals.

I Somoskövi Á, Zissel G, Zipfel P, Ziegenhagen M, Klaucke J, Haas H, Schlaak M, Müller-Quernheim J: Different Cytokine Patterns Correlate with the Extent of the Disease in Pulmonary Tuberculosis. European Cytokine Network 1999. (Accepted for publication)

II Somoskövi Á, Győri Zs, Czoboly N, Magyar P. Application of a computer-directed automated microscope in mycobacteriology. Int J Tuberc Lung Dis 1999; 4:354-357.

III Somoskövi Á, Magyar P. Comparison of the Mycobacteria Growth Indicator Tube with MB Redox, Löwenstein-Jensen and Middlebrook 7H11 media for recovery of mycobacteria in clinical specimens. J Clin Microbiol 1999; Vol 37. No.5

IV Somoskövi Á, Magyar P: Mycobacterium tuberculosis complex kimutatása Accuprobe nukleinsav hibridizációs próba segítségével Bactec 12B folyékony táptalajból. Medicina Thoracalis 1997; 50:9-13.

V Somoskövi Á, Tolnay E, Vajda E, Zsiray M, Tarján E, Magyar P: Mycobacterium tuberculosis laboratóriumi gyorsdiagnosztikája polimeráz láncreakció segítségével. Medicina Thoracalis 1995; 48:3-9.

1. Introduction:

1.1. General Introduction (I):

Tuberculosis results from an infection with *Mycobacterium tuberculosis* (*M. tuberculosis*). In patients with tuberculosis, the infection with the causative pathogen leads to the accumulation and activation of alveolar macrophages and lymphocytes that release different regulatory mediators, which results in T helper lymphocyte/macrophage alveolitis (Gergely, 1998; Hutás, 1998; Schluger and Rom, 1998). The immune response to tuberculosis is a double-edged weapon that can contribute both to clearance of the infection and to tissue damage. The course of the disease can be influenced by the cytokine pattern elicited by *M. tuberculosis* or the regulatory mechanisms of the host, as demonstrated in a recent study where a correlation was found between the strength of the T helper 1 and T helper 2-type cytokine release of bronchoalveolar lavage cells and the radiographic severity of the disease (Somoskövi et al., 1999C).

The World Health Organization estimates that as many as one-third of the world's population have been infected with *M. tuberculosis*. Each year, there are 8-10 million new cases of tuberculosis and about 3 million deaths due to it. At present, tuberculosis is the leading cause of death from a single infectious agent in adults and accounts for approximately 25% of all preventable adult deaths in the world. In addition, about 80% of all tuberculosis cases affect persons of child-bearing age or during their most economically productive years (ages 15-59) (WHO Fact Sheet No. 93, 1995; Snider et al., 1994).

In Hungary, after 20 years of decline, between 1990 and 1995 the incidence of tuberculosis displayed an increase of almost 25% (rising from 31 per 100 000 to 39 per 100 000) (Pulmonológiai Intézetek éves

epidemiológiai és működési adatai, 1990-1995; Hutás, 1998). In 1998, the annual incidence of pulmonary tuberculosis was 39.2 per 100 000, 44.3% of the cases proving culture-positive (Pataki, 1999). Although, the rate of recurrance was 13% of all cases and the rate of culture-positive cases was not higher than in previous years, the situation was worsened by the fact that the number of patients with incurable disease did rise. In 1995, we lost 11 patients with tuberculosis shortly after admission to our department, and 9 of these patients died within 24 hours (Hutás, 1998).

Laboratory methods play a crucial role in establishing the diagnosis of tuberculosis, monitoring the therapy and preventing transmission of the disease, because the clinical signs and symptoms of the disease are not specific. Abnormal chest radiographs may strongly suggest the presence of tuberculosis, but other lung diseases can give rise to similar findings. In addition, chest radiographs cannot distinguish between current active tuberculosis and previous infection. Laboratory results must be not only accurate, but also rapidly available. However, the laboratory techniques most commonly employed worldwide for the diagnosis of tuberculosis were developed in the last century and have been modified only slightly subsequently. Thus, the recent upsurge of tuberculosis has highlighted the shortcomings of the laboratory diagnosis of tuberculosis. It is generally accepted that the use of a combination of liquid and solid media is the gold standard for the isolation of mycobacteria (Tenover et al., 1993; Styrt et al., 1997). The past few years have brought significant changes to clinical mycobacteriology with the introduction of broth-based cultivation and molecular biological methods. However, the value of any new method can be assessed only in comparison with conventional techniques currently available in clinical laboratories.

1.1.1. Microscopy:

Acid-fast microscopy is still the easiest, cheapest and most rapid procedure in mycobacteriology. It has a specificity of over 99% (for

Mycobacteria sp.), it requires standard reagents with long shelf-lives and it can be applied successfully in any laboratory (Smithwick, 1976; Roberts et al., 1991). However, the sensitivity of microscopy is often not satisfactory. When a single smear of a respiratory specimen is examined, acid-fast microscopy furnishes false-negative results in between 25 and 50% of patients with active tuberculosis (Kim et al., 1984; Gordin and Slutkin, 1990). Thus, while a positive smear test is useful in making a presumptive diagnosis of tuberculosis, a negative test does not rule out the presence of the disease. Nor does microscopic examination allow a differentiation between viable and non-viable bacteria, or the identification of different mycobacteria species. It has recently been shown that the concentration of sputum by cytocentrifugation results in an increased detection of mycobacteria in clinical specimens. However, the method requires expensive instrumentation and only a limited number of smears can be processed, which leads to a delay in the reporting of the results (Saceanu et al., 1993; Fodor, 1995). The staining of smears with a fluorochrome (e.g. auramine) is superior to the classical Ziehl-Neelsen (ZN) or Kinyoun method, since a lower magnification can be used with fluorescence microscopy, which reduces the time demanded for screening. More importantly, with a fluorochrome dye, mycobacteria seen as bright staining against a dark background are easier to detect. Hence, smears examined by this method have been found to have a greater sensitivity than those stained by the ZN method (Strumpf et al., 1979; Sommers et al., 1983). Unfortunately, good laboratory practice requires the confirmation of any fluorescence microscopy smear-positive results by a ZN stain (Salfinger and Pfyffer, 1994; Hanna, 1996). Thus, the initial advantage of the faster detection of acid-fast bacteria (AFB) by fluorescence microscopy is reduced. Regardless of the staining technique, it is especially important for laboratory staff to be vigilant when examining acid-fast smears, to avoid any false-negative report. However, the careful examination of smears is time-consuming and labour-intensive in laboratories with very high daily volumes. It is clear that, while acid-fast microscopy is an

important adjunct to the detection of *M. tuberculosis*, it is not an adequate criterion alone and must be followed by culture.

1.1.2. Culture:

The yield of cultivation for the detection of *M. tuberculosis* is higher than that of acid-fast microscopy. Historically, the egg-based media, such as that of Löwenstein-Jensen (LJ), are the best-known of the solid media used for the isolation of *M. tuberculosis*. The agar-based media, e.g. Middlebrook 7H10 and 7H11 agar, offer a better opportunity for the examination of colonial morphology and the detection of mixed cultures than do egg-based media (Middlebrook and Cohn, 1958; Cohn et al., 1968). However, on conventional solid media, colonies of *M. tuberculosis* are rarely visible before 6-8 weeks of incubation (Nolte and Metchcock, 1995). The identification and determination of the drug susceptibility of a mycobacterium isolate by culturing can add 4-6 weeks to this already long process. It can be 2-3 months before the results of identification and drug susceptibility tests are available (Heifets and Good, 1994; Smithwick, 1994). Moreover, it has been observed that as many as 29.5% of patients reported to have tuberculosis are not culture-confirmed when solid media are used alone (Nardell et al., 1995). The use of a liquid medium is generally recommended for subcultures and *in vitro* tests. A liquid medium can be used for the primary isolation of *M. tuberculosis* from sputum only if it is a selective medium (containing incorporated antibiotics) (Kent and Kubica, 1985). In selective liquid media, the growth of *M. tuberculosis* can be detected within 1 to 2 weeks, depending on the number of bacteria in the specimen (Morgan et al., 1983; Roberts et al., 1983). The most reliable medium of this type is Middlebrook 7H12 broth, manufactured as Bactec 12B vials for the rapid detection of growth in the Bactec 460 TB system (Becton-Dickinson Diagnostic Instrument Systems, Sparks, Md., U.S.A.) (Middlebrook et al., 1977). Although affording a better recovery of mycobacteria and a decreased detection time, the Bactec system is radioactive and laborious. Furthermore, even if no growth is detected in

the Bactec 12B vials, the final report that the specimen is culture-negative can be issued only after 8 weeks of incubation of the LJ slants. Further development and introduction of new cultures and other direct detection methods are therefore necessary for the more rapid, more reliable and less labour-intensive detection of mycobacteria.

1.2. Evaluation of a self-developed computer-directed microscope for acid-fast microscopy (II):

Acid-fast microscopy is usually the first bacteriological test for confirmation of the diagnosis of tuberculosis and for the rapid identification of potentially infectious patients. However, the method has limitations. The major drawback is the relatively low sensitivity as compared with culture. The sensitivity of microscopy is influenced by factors such as:

- the bacterial load (positivity requires a concentration of at least 10 000 bacteria/ml in specimens),
- the proportion of patients with severe tuberculosis,
- the lower volume of the specimen examined as compared with culture,
- the quality of the examination (Smithwick, 1976; Heifets and Good, 1994).

It is recommended that a negative result should be reported only following the examination of at least 100, but preferably 300 microscopic immersion viewfields, and all microscopic results should be reported to the physician within 24 hours of specimen collection (Smithwick, 1976; Heifets and Good, 1994). Consequently, the examination is time-consuming and laborious. This may lead to false-negativity, with the consequence that the physician may not take the necessary control and prevention steps and may consider another (mis)diagnosis. In order to increase the efficacy of microscopy, we have constructed a model automated microscope capable of detecting the presence of mycobacteria in smears of clinical specimens.

In liquid media, the detection of the presence of mycobacteria is based on their metabolism rather than on visible colonies. Growth positivity should therefore be confirmed by microscopy. This involves extra work for the laboratory staff, a longer time before the reporting of growth positivity and the performance of identification tests. Accordingly, besides assisting the direct manual method, we also intended to improve the confirmatory step of liquid medium growth positivity.

1.3. Evaluation of the manual MGIT and MB Redox systems versus conventional solid media (III):

The definitive diagnosis of tuberculosis is dependent on the isolation, identification and drug susceptibility testing of the causal agent *M. tuberculosis* by cultivation. Although cultivation is specific on a solid medium such as that of LJ or Middlebrook agar, it is insensitive and rather slow (Smithwick, 1994; Nolte and Metchcock, 1995). The introduction of the broth-based Bactec 460 TB system has considerably enhanced the recovery of mycobacteria and the time to detection has decreased (Morgan et al., 1983; Anargyros et al., 1990; Abe et al., 1992). The main limitations of the system are the high cost of disposal of the radioactive waste and the need for instrumentation.

To circumvent these limitations, two non-radioactive broth-based culture methods were recently developed, known commercially as the Mycobacteria Growth Indicator Tube (MGIT; BBL Becton-Dickinson Microbiology Systems, Cockeysville, Md., U.S.A.) and the MB Redox (Biotest AG, Dreieich, Germany). MGIT contains a modified Middlebrook 7H9 broth with an oxygen quenching-based fluorescent sensor. The large amount of oxygen initially dissolved in the broth quenches fluorescence, but the growth of microorganisms present, such as mycobacteria, is accompanied by the consumption of oxygen, which allows the indicator to

fluoresce under 365 nm UV light. MB Redox utilizes a modified Kirchner medium with a colourless tetrazolium salt as growth indicator. During bacterial growth, this tetrazolium salt is reduced to a formazan, the colour of which indicates the presence of mycobacteria.

In previous studies, MGIT has been reported to have a sensitivity, a rapidity and recovery rates comparable to those of Bactec 460 TB (Casal et al., 1997; Pfyffer et al., 1997). MB Redox has also been found to be similar in effectivity to Bactec 460 TB, but as yet only one report appears to be available in this respect (Naumann et al., 1996). In this report, the time to detection of mycobacteria in smear-positive and negative specimens was not given.

In order to define any differences between these two recently introduced media, the aim of the present study was to compare MGIT and MB Redox both with each other and with the reference LJ and Middlebrook 7H11 media as concerns recovery rates, the mean time required for the detection of mycobacteria in clinical specimens, and the contamination rates.

1.4. Evaluation of the automated MB/BacT system versus the semi-automated Bactec 460 TB system and solid medium:

The recommendation of the experts at the Centers for Disease Control and Prevention (Denver, Co., U.S.A.) is to use both liquid and solid media in order to detect mycobacterial growth within 14 days of specimen inoculation (Tenover et al., 1993; Styrt et al., 1997). For many years, the only culture system with the potential to provide this target turnaround time was the Bactec 460 TB system. In this system, processed specimens are inoculated into vials which contain a radiolabelled substrate, ^{14}C-palmitic acid. During mycobacterial growth ^{14}C-labelled CO_2 is produced

and released into the air of the vials. The Bactec 460 TB equipment allows measurement of this radioactive gas and reports in terms of a growth index (GI). In general, specimens are read twice weekly for the first 2 weeks and once a week thereafter for a total of 6 weeks. Once the GI reaches a value of 10 or greater, the vials should be read on a daily basis. Disadvantages include the manual loading of the racks containing the vials and the need to establish a reading schedule. Thus, this system requires a considerable amount of work and attention to the safety and regulatory issues associated with the use of radioisotopes. For these reasons, a technically more efficient, non-radiometric mycobacterial culture system is desirable. Previous results have proved that the recently introduced MGIT and MB Redox systems are suitable non-radiometric alternatives to Bactec (Palaci et al., 1996; Casal et al., 1997; Naumann et al., 1997; Pfyffer et al., 1997; Somoskövi et al., 1999B) However, these methods still require manual processing and are particularly optimal for laboratories which cannot afford or, due to the low number of processed specimens, do not need instrumentation.

The recently developed MB/BacT (Organon Teknika, Turnhout, Belgium) system is a fully automated, continuous monitoring system, which employs a colorimetric sensor and reflected light to monitor the production of CO_2 dissolved in the culture medium. If mycobacteria are present, CO_2 is produced as the organisms metabolize substrates in the culture medium. When the growth of mycobacteria produces CO_2, the colour of the gas-permeable sensor at the bottom of each vial changes to dark-green to lighter-green or yellow. The lighter colours result in an increase in the reflectance units monitored by the system. The measured values are transmitted and recorded by a computer every 10 minutes. We compared MB/BacT with the reference Bactec 460 TB system and LJ, and evaluated the performance of this new equipment in terms of sensitivity, time to detection of *M. tuberculosis*, handling requirements and degree of automation.

1.5. Evaluation and introduction of the Amplicor polymerase chain reaction assay for the routine direct detection of *M. tuberculosis*:

In order to meet the aim of controlling the spread of tuberculosis, laboratory services must be completed in a shorter time and with the introduction of more accurate methods. The goal of research in rapid diagnostics, therefore, is to develop reliable procedures that can detect and identify mycobacteria directly in clinical specimens and avoid the many weeks required for isolation and identification by cultivation. The greatest breakthrough came from biotechnology with the application of nucleic acid amplification techniques. Gene amplification can achieve the goals of reducing the generation time of mycobacteria to minutes and of replacing biological growth on artificial media by enzymatic reproduction of nucleic acid *in vitro*. Since its first application to the diagnosis of tuberculosis (Brisson-Noel et al., 1989), the polymerase chain reaction (PCR) has become the most widely-used technique for amplifying nucleic acids from mycobacteria. Diagnostic PCR is a deoxyribonucleic acid (DNA) amplification technique that uses specific DNA sequences to serve as markers for the presence of *M. tuberculosis* and is, in theory, capable of detecting a single organism in a biological specimen such as sputum, lavage fluid or gastric juice (Persing, 1993). In the PCR technique, a specimen that may contain *M. tuberculosis* is first heated to denaturate double-stranded DNA. After this, specific synthetic oligonucleotides, primers, bind to the target DNA sequences of *M. tuberculosis* that are unique to it and a heat-stable DNA polymerase then extends the primers to create a complete and complementary strand of DNA. This process is typically repeated sequentially and millions of copies of the target DNA sequence are created (Saiki et al, 1985; Mullis and Faloona, 1987). These amplified products can then be easily detected by gel electrophoresis or hybridization to capture probes (Shawar et al., 1987; Persing, 1993; Kox et al., 1996; Stauffer et al., 1997). If the target organism is not present in

the sample being examined, the primers have nothing to bind to and no amplification occurs.

For any assay to be useful in a clinical diagnostic setting, a careful optimization of the amplification conditions and standardization of the protocols and reagents are needed to ensure proper performance. Results from a recent study comparing the performances of seven clinical laboratories highlight the laboratory-to-laboratory variability that can occur when PCR is performed by individual laboratories using different tests and non-standardized reagents and protocols (Noordhoek et al., 1993). In the recently commercially developed Amplicor MTB PCR assay (Roche Molecular Systems Inc., Branchburg, NJ, U.S.A.), reagents are standardized and quality controlled to ensure performance. 2'-Deoxyuridine-5'-triphosphate (dUTP) and uracil-N-glycosylase have also been incorporated into the assay to prevent false-positive results from carry-over contamination (Longo et al., 1990). The current assay targets a mycobacterium genus-specific gene encoding the 16S ribosomal ribonucleic acid (rRNA) (Böddinghaus et al., 1990). *M. tuberculosis* is identified by hybridizing the amplification products to a species-specific probe (Tevere et al., 1996). The purpose of our study was to compare the Amplicor PCR test for the direct detection of *M. tuberculosis* in respiratory specimens with conventional culture and staining techniques.

2. Aims of the study:

I To develop a model of an automated and computer-directed system in order to improve the efficacy of acid-fast microscopy.

II Modern culture techniques: The introduction of new broth-based culture systems for the primary isolation of *M. tuberculosis* at the Laboratory for Mycobacteria of the Department of Respiratory Medicine at Semmelweis University of Medicine:

II/1 Evaluation of the manual MGIT and MB/Redox systems versus conventional solid media.
II/2 Evaluation of the automated MB/BacT system versus the semi-automated Bactec 460 TB system and solid medium.

III Molecular biological methods: Evaluation and introduction of the Amplicor PCR assay for the routine direct detection of *M. tuberculosis* at the Laboratory for Mycobacteria of the Department of Respiratory Medicine at Semmelweis University of Medicine.

3. Materials and Methods:

3.1. Evaluation of a self-developed computer-directed microscope for acid-fast microscopy (II):

Specimen processing, and manual and automated smear examination were performed as reported previously (Somoskövi et al., 1999A).

3.2. Evaluation of the manual MGIT and MB Redox systems versus conventional solid media (III):

Specimen collection, specimen processing, inoculation, cultivation, quality control, identification and statistical analysis were performed as described previously (Somoskövi et al., 1999B).

3.3. Evaluation of the automated MB/BacT system versus the semi-automated Bactec 460 TB system and solid medium:

3.3.1. Specimens:

A total of 200 specimens from 137 patients, submitted for the detection of mycobacteria between November 1996 and January 1997, were evaluated: 158 sputum specimens and 42 bronchial washings. All these specimens were digested and decontaminated by the N-acetyl-L-cysteine / NaOH method (Kubica et al., 1963). A 4% concentration (starting concentration) of NaOH was used. After decontamination, smears were prepared from the concentrated sediments of the specimens for ZN acid-fast staining (Smithwick, 1976; Fodor, 1984).

3.3.2. Culture and identification (IV):

An aliquot of the processed specimen was inoculated onto each culture medium as follows: 0.5 ml into an MB/BacT process vial, 0.5 ml into a Bactec 12B vial and 0.2 ml onto each of two LJ slants. Prior to inoculation of the liquid media with the specimen, the respective manufacturer's

antibiotic supplement was added to MB/BacT (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin; MB/BacT Antibiotic Supplement) and Bactec 12B (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin; PANTA). MB/BacT vials were placed into the MB/BacT instrument and incubated at 37 °C, with monitoring for mycobacterial growth as described above. The MB/BacT cultures were incubated for 7 weeks or until signalled by the instrument as positive. Bactec 12B vials were incubated at 37 °C and monitored for growth by the Bactec 460 TB instrument. In general, vials were monitored for growth twice weekly for the first 2 weeks and once a week thereafter for a 6 weeks. If no growth had occurred in the MB/BacT or Bactec 12B vials by the end of the incubation period, the culture was considered negative for mycobacterial growth. When an MB/BacT vial was signalled as positive, and for Bactec 12B when the GI reached 100, a sample of the broth was removed and used to prepare a ZN smear and for subculture on a LJ slant. All solid media were incubated at 37 °C and inspected weekly for 8 weeks or until mycobacterial colonies were detected. Identification tests were performed on colonies growing on a solid medium or an aliquot of broth removed from a positive MB/BacT or Bactec 12B vial. Methods used for identification included conventional biochemical tests and nucleic acid hybridization (Gen-Pobe Inc., San Diego, Calif., U.S.A.) (Kent and Kubica, 1985; Nolte and Metchcock, 1995; Somoskövi et al., 1997B).

3.3.3. Statistical analysis:

The χ^2 test was used to evaluate differences between recovery and contamination rates in different media, the Primer for Biostatistics program (version 3.01, McGraw Hill, U.S.A., 1992) being employed.

3.4. Evaluation and introduction of the Amplicor polymerase chain reaction assay for the routine direct detection of *M. tuberculosis*:

3.4.1. Specimen collection and processing (IV):

Specimens were collected from patients suspected of having tuberculosis or who were being monitored for treatment with antituberculosis drugs. A total of 1869 respiratory specimens from 543 patients were investigated. Specimens were limited to expectorated and induced sputa, bronchoalveolar lavages and bronchial washings. Specimens were decontaminated and digested by the the N-acetyl-L-cysteine / NaOH method (Kubica et al., 1963). A 4% concentration of NaOH was used (starting concentration). After decontamination, smears were prepared from the concentrated sediments of the specimens for ZN acid-fast staining (Smithwick, 1976). For each specimen, two LJ slants were inoculated with 0.2 ml of specimen. Cultures were incubated at 37 °C and examined weekly for a total of 8 weeks. Isolates were identified by standard procedures (Kent and Kubica, 1985; Nolte and Metchcock, 1995; Somoskövi et al., 1997B). The residual processed specimens were stored at -20 °C until Amplicor PCR was performed.

3.4.2. Amplicor PCR assay (V):

The Amplicor PCR assay includes three major steps: PCR target amplification, hybridization of the amplified product to a specific nucleic acid probe and colorimetric detection.

Genus-specific primers located in a highly conserved region of 16S rRNA *Mycobacterium* gene are used to amplify a 584-bp sequence (Böddinghaus et al., 1990; Tevere et al.,1996). Carry-over contamination in the Amplicor PCR test is prevented by incorporating dUTP in place of 2'-deoxythymidine-5'-triphosphate during amplification and utilization of uracil-N-glycosylase (AmpErase) to enzymatically cleave the uracil-containing DNA strands left from previous reactions prior to thermal cycling (Longo et al., 1990). This enzyme is subsequently inactivated at

the temperatures used during thermal cycling. Specimens were prepared by adding 100 µl of concentrated specimen to 500 µl of sputum wash solution and were centrifuged at 12 500 x g for 10 minutes. The pellet was resuspended in 100 µl of the lysis reagent, vortexed, heated for 45 minutes at 60 °C, and neutralized by the addition of 100 µl of neutralization reagent. For amplification, 50 µl of neutralized specimen was added to an equal volume of master mix with AmpErase and amplified by using a TC-9600 thermal cycler (Perkin-Elmer, Norwalk, Conn., U.S.A.) as described previously (Somoskövi et al., 1995). After addition of 100 µl of denaturation solution, the PCR tubes were incubated for 10 minutes at room temperature and 25 µl of denatured amplicons was added to each well of the MTB probe-coated microwell plate containing 100 ml of hybridization buffer. After 90 minutes of hybridization at 37 °C, the microwells were washed five times with wash buffer, 100 µl of avidin-horseradish peroxidase conjugate was added and the plates were reincubated for 15 minutes at 37 °C and rewashed five times. For colorimetric detection, 100 µl of tetramethylbenzidine peroxide solution was added, the wells were covered and incubated for 10 minutes at room temperature, and the reaction was stopped with 100 µl of stop reagent. The A450 was read with a Mios Junior microplate reader (Merck Ltd.). An A450 of > 0.35 was scored as a positive result and an A450 of < 0.35 as a negative result. In each independent assay, PCR test results were compared with results for one positive and three negative controls. Controls were included in the kit.

3.4.3. Discrepancy analysis:

In those cases in which culture results were discrepant from the Amplicor PCR results, clinical data and other results obtained with additional non-study specimens from the patient were considered. Assessment of each patient's clinical picture included the patient's history, symptoms, chest X-ray, tuberculin skin test result and history of drugs administered, whenever these data were available.

3.4.4. Statistical analysis:

Statistical comparisons between Amplicor PCR and culture data were performed by using χ^2 analysis, the Primer for Biostatistics program (version 3.01, McGraw Hill, U.S.A., 1992) being employed.

4. Results:

4.1. Evaluation of a self-developed computer-directed microscope for acid-fast microscopy (II):

A total of 132 smears of sputum and 74 smears of liquid media were examined in parallel by manual and automated microscopy. Conventional microscopy detected 53 positive and 79 negative smears of sputum, while automated microscopy detected 55 positive and 77 negative smears of sputum. Fluorescent smear positivity could be confirmed by ZN restaining and Bactec and LJ culture in all samples. From the 74 smears of liquid media, 50 were found positive by both the automated and the manual version. The positivity of these smears was confirmed by LJ subculture. In the remaining 24 smears, automated microscopy did not detect any AFB, while the ZN smears revealed non-mycobacterial contamination.

4.2. Evaluation of the automated MB/BacT system versus the semi-automated Bactec 460 TB system and solid medium:

A total of 200 respiratory specimens were cultivated in liquid (MB/BacT and Bactec 12B) and on solid (LJ) media. Cultures positive for AFB were obtained for 55 specimens, of which 10 were smear-positive. The mycobacterial isolates included *M. tuberculosis* (n = 48), *M. xenopi* (n = 6) and *M. chelonei* (n = 1).

Focusing on each type of cultivation system separately, MB/BacT and Bactec 12B detected 89.6% and 97.9%, whereas LJ detected 79.2% of all *M. tuberculosis* isolates (Table 4.2.1). Although both liquid media were more sensitive than conventional LJ, the statistical analysis revealed a significant difference only between Bactec 12B and LJ ($p = 0.01$). Each of the three media detected all 10 smear-positive specimens (all contained

M. tuberculosis). In smear-negative specimens, the rates of recovery of *M. tuberculosis* were 86.8% and 97.3% with MB/BacT and Bactec 12B, as compared with 73.6% with LJ. Some isolates grew only on a single medium: MB/BacT alone detected 3 additional isolates of *M. tuberculosis* while Bactec 12B alone detected 4 additional isolates of *M. tuberculosis*.

Table 4.2.1.

Rates of recovery of mycobacteria, *M. tuberculosis* and contaminants for MB/BacT, Bactec 12B and LJ.

Media	All isolates n = 55 n / %	*M. tuberculosis* n = 48 n / %	Contaminant n = 27 n / %
MB/BacT	45 (81.8)	43 (89.6)	15 (7.5)
Bactec 12B	52 (94.5)	47 (97.9)	8 (4.0)
LJ	42 (76.3)	38 (79.2)	4 (2.0)

χ^2 test for differences in recovery of mycobacteria and *M. tuberculosis*: Bactec 12B vs.LJ, p = 0.01, significant.

χ^2 test for differences in contamination: no significant differences between liquid media.

As concerns the recovery rates on liquid and solid media in combination (gold standard), MB/BacT plus LJ (combination A) recovered 93.8% of all *M. tuberculosis* isolates, while Bactec 12B plus LJ (combination B) yielded 97.9% (Table 4.2.2). There was no statistical difference between the two gold standards. Although the combination of the broths (MB/BacT plus Bactec 12B; combination C) recovered all the *M. tuberculosis* isolates, the difference in comparison with combination A or B was not statistically significant. However, each combination reached a significantly higher level of sensitivity in comparison with LJ ($p<0.05$).

Table 4.2.2.

Rates of recovery of mycobacteria and *M. tuberculosis* for liquid and solid media in combination.

No. of isolates	Combination A n (%)	Combination B n (%)	Combination C n (%)
Mycobacteria n = 55	51 (92.7)	52 (94.5)	55 (100)
M. tuberculosis n = 48	45 (93.8)	47 (97.9)	48 (100)

χ^2 test for differences in recovery of mycobacteria and *M. tuberculosis*: combination A, B, or C vs.LJ, $p<0.05$, significant.

Overall, the mean times to detection for all *M. tuberculosis* isolates were 16.3, 12.5 and 27 days in MB/BacT, in Bactec 12B and on LJ (Table 4.2.3). *M. tuberculosis* was detected in smear-positive specimens after a mean of 12.3 days in MB/BacT, 7.3 days in Bactec 12B, and 25 days on LJ (Table 4.2.3). For smear-negative specimens, the mean times to detection of *M. tuberculosis* were 17.4 days, 13.7 days and 27.5 days for MB/BacT, Bactec 12B and LJ (Table 4.2.3). Microorganisms other than mycobacteria contaminated 7.5% of MB/BacT, 4% of Bactec 12B and 2% of LJ (Table 4.2.1). Statistical analysis did not reveal a significant difference between the two liquid media. The low number of non-tuberculous mycobacteria (NTM) did not allow a detailed evaluation of this group.

Table 4.2.3.

Mean time to detection of *M. tuberculosis* in clinical specimens.

Media	All *M. tuberculosis* days (range)	*M. tuberculosis* smear-positive days	*M. tuberculosis* smear-negative days
MB/BacT	16.3 (8-39)	12.3	17.4
Bactec	12.5 (6-27)	7.3	13.7
LJ	27.0 (14-51)	25.0	27.5

4.3. Evaluation and introduction of the Amplicor polymerase chain reaction assay for the routine direct detection of *M. tuberculosis*:

Mycobacteria were cultured from 197 of the 1869 specimens: 188 were identified as *M. tuberculosis* and 9 were identified as NTM. AFB could be detected by microscopy in 48 specimens (all contained *M. tuberculosis*). The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the Amplicor PCR test are given in Table 4.3.1.

Table 4.3.1.

Sensitivity, specificity, PPV and NPV of the Amplicor PCR test and ZN microscopy vs. LJ cultivation.

	Culture +	Culture -	Sensitivity	Specificity	PPV	NPV
PCR+	140	120	74.8%	92.8%	53.8%	97.0%
PCR-	48	1552				
ZN+	44	4	23.4%	99.7%	91.6%	92.0%
ZN-	144	1668				

As compared with those of culture, the sensitivity of the Amplicor PCR was 74.5% and the specificity was 92.8%, while the ZN stain had a sensitivity of 23.4% and a specificity of 99.7%. Forty-eight specimens of 40 patients, all receiving therapy for tuberculosis, were Amplicor PCR-negative and culture-positive. Thirty-two of these specimens contained fewer than 10 colony formation units (CFU) of *M. tuberculosis* on a LJ slant. These 48 specimens were retested before and after the addition of 15 μl of the positive control (spike back-testing) to determine whether amplification inhibitors were present. Twenty specimens were positive on retesting, indicating a sampling error, 12 specimens were negative on retesting and did not inhibit the amplification of the positive control, and 16 specimens were found to inhibit the amplification of the positive control. For specimens which were ZN-positive, the sensitivity of the Amplicor PCR was 100%; for ZN-negative specimens, the sensitivity was 66.6%.

False-positive Amplicor PCR results occurred in 120 specimens (including the 4 specimens that were ZN-positive and culture-negative) of 84 patients. Discrepant analysis revealed that 108 specimens were obtained from patients with either a positive history of tuberculosis (20 patients), a previous isolate (44 patients) or a previously positive PCR (8 patients) result. These specimens were considered true-positives which were missed by culture, while the remaining 12 specimens were considered false-positives. When these confirmed results were taken into consideration, the initial results were recalculated as summarized in Table 4.3.2. The resolved, clinical sensitivity, specificity, PPV and NPV were 83.8%, 99.2%, 95.4% and 97.0%, respectively, for Amplicor PCR, 63.5%, 100%, 100% and 93.5%, respectively, for culture, and 16.2%, 100%, 100% and 86.3%, respectively, for ZN. Statistical analysis revealed a significant difference in sensitivity between Amplicor PCR, culture and ZN ($p<0.01$). The clinical sensitivity of the Amplicor PCR test for ZN-positive specimens was 100%, while for ZN-negative specimens it was 80.6%.

Table 4.3.2.

Clinical sensitivity, specificity, PPV and NPV of the Amplicor PCR test, LJ cultivation and ZN microscopy after discrepancy analysis.

	Confirmed +	Confirmed -	Sensitivity	Specificity	PPV	NPV
PCR +	248	12	83.8%	99.2%	95.4%	97.0%
PCR-	48	1552				
Culture +	188	0	63..5%	100%	100%	93.5%
Culture -	108	1564				
ZN +	48	0	16.2%	100%	100%	86.3%
ZN -	248	1564				

5. Discussion:

5.1. Evaluation of a self-developed computer-directed microscope for acid-fast microscopy (II):

Our results showed that the model computer-directed microscopy could readily detect the presence of AFB in either clinical specimens or liquid media. It is important to emphasize that the software does not determine positivity, but offers all archived material for a final decision to an expert. Up to 20 archived pictures can be reviewed in parallel to decrease the examination time. Preparation and careful examination of a smear for 300 microscopic immersion viewfields manually requires about 30 min (Pfyffer et al., 1996). Examination of a smear (for 100 fluorescent viewfields) with the automated microscope was more rapid than with the conventional method, requiring only about 1.8-2.0 min per slide. It may be speculated that the time-saving could allow the examination of parallel smears from the same specimen, similarly as in cultivation, which could well increase the sensitivity. In contrast with the manual method, the instrument always examines the required number of viewfields according to standard recommendations, and each of them with the same care. The possibility of a false-negative report is greatly decreased, since the effects of tiredness are excluded, and the standardized microscopic examination procedure can be followed. Further, no dark-room is needed, so there is no eye stress due to fluorescence microscopy, which can similarly lead to false-negativity.

We found that the automated microscope could detect AFB in all smears of AFB-positive liquid media, which supports its application for the automation and speeding-up of both the direct smear examination and the confirmatory step of growth positivity of liquid media. We also demonstrated that a walk-away automated system may decrease the chance of false-negative acid-fast microscopy, help prevent delay in the

reporting of growth positivity or in the starting of identification tests, free laboratory staff for other duties, and afford a possibility for rapid and reliable screening of the population at risk in large numbers.

5.2. Evaluation of the manual MGIT and MB Redox systems versus conventional solid media (III):

A total of 486 clinical specimens received for routine mycobacterial cultivation were processed. Of these 117 (24.1%) specimens were AFB-positive, of which 22 (18.8%) were smear-positive and 95 (81.2%) were smear-negative. The mycobacterial species identified were *M. tuberculosis* (n = 112), *M. xenopi* (n = 3) and *M. kansasii* (n = 2).

In our study, MGIT yielded the highest recovery rate of *M. tuberculosis*, but both liquid media (81.3% with MGIT and 72.3% with MB Redox) were better than the solid media (64.3% with LJ and 60.7% with Middlebrook 7H11), in accord with other reports (Naumann et al., 1996; Casal et al., 1997; Pfyffer et al., 1997; Rivera et al., 1997) (Table 5.2.1). In contrast with the findings of Casal et al. (1997) and Pfyffer et al. (1997), the differences between MGIT and the solid media were statistically significant. However, while Pfyffer et al. (1997) detected 70 (38.9%) smear-positive specimens and Casal et al. (1997) detected 174 (82.1%) smear-positive specimens, in our study there were only 22 (18.8%) smear-positive specimens. Their higher rates of smear-positive specimens may have reduced the difference between MGIT and the solid media in the rate of recovery. Thus, a statistically significant level was not attained in their studies. Our results were also in contrast with the findings of Naumann et al. (1996), as our statistical analysis of the recovery rates of *M. tuberculosis* did not reveal significant differences between MB Redox and the solid media. However, the statistically significant difference between MB Redox and solid media observed by Naumann et al. (1996) may have been due to the lower number of their isolates (50 *M. tuberculosis* and 16 NTM isolates in 974 specimens).

Table 5.2.1.

Rates of recovery of mycobacteria and contaminants for MGIT, MB Redox, LJ and Middlebrook 7H11.

Media	All isolates n = 117 n (%)	*M. tuberculosis* n = 112 n (%)	NTM n = 5 n (%)	Contaminant n = 24 n (%)
MGIT	93 (79.5)	91 (81.3)	2 (40)	21 (4.3)
MB Redox	84 (71.8)	81 (72.3)	3 (60)	19 (3.9)
LJ	73 (62.4)	72 (64.3)	1 (20)	10 (2.1)
M-7H11	68 (58.1)	68 (60.7)	0	13 (2.7)

χ^2 test for differences in recovery of mycobacteria and *M. tuberculosis*: MGIT vs. solid media, $p<0.01$, significant; MB redox vs. Middlebrook-7H11, $p<0.05$, significant.

χ^2 test for differences in contamination: no significant differences.

MGIT detected all 22 smear-positive specimens, while the other three media each detected 21 of the 22 smear-positive specimens. All smear-positive specimens contained *M. tuberculosis*. For the smear-negative specimens, the *M. tuberculosis* recovery rates were 76.6% with MGIT, 66.6% with MB Redox, 56.6% with LJ and 52.2% with Middlebrook 7H11. A statistically significant difference was found between MGIT and LJ ($p<0.01$) and between MGIT and Middlebrook 7H11 ($p<0.05$). The difference between MB Redox and Middlebrook 7H11 was close to being statistically significant ($p = 0.069$). Although there was no statistically significant difference between the two liquid media, our results indicate that the MGIT method may be more sensitive than MB Redox.

It is important to note that none of the four media detected all of the mycobacterial isolates. The yield of mycobacterial isolates increased with

the number of media used in combination, as observed by others (Pfyffer et al., 1997; Rivera et al., 1997). MGIT provided 14 more, and MB Redox 9 more mycobacterial isolates when in combination with the two solid media. Our data indicate a need for the inclusion of a solid medium in the primary isolation, because 6 mycobacterial isolates grew only on LJ and 2 only on Middlebrook 7H11. However, the workload, the financial resources and the restricted amount of sediment of a clinical specimen are limiting factors in work with too many different types of media.

Recovery rates were also compared on media in different combinations. For MGIT plus MB Redox (combination A) the recovery rate was 92.9%, for MGIT plus LJ plus Middlebrook 7H11 (combination B) it was 94.6%, for MB Redox plus LJ plus Middlebrook 7H11 (combination C) it was 88.4% and for LJ plus Middlebrook 7H11 (combination D) it was 75.0% of all the *M. tuberculosis* isolates (Table 5.2.2). The fact that no statistical difference was found between the two gold standards as concerns the recovery rates of *M. tuberculosis*, and the observation that these recovery rates compare well with those obtained with the Bactec 12B medium, suggest that either combination could replace the Bactec 12B plus solid media standard (Abe et al., 1992; Pfyffer et al., 1997). It was recently demonstrated that a combination of two liquid media (MGIT and Bactec 12B) was more efficient than combinations of liquid and solid media (Pfyffer et al., 1997). Those authors also recommended determination of the efficacy of combined liquid media which do not contain radioisotopes. In our study, the combination of MGIT and MB Redox (combination A) displayed a slightly lower recovery rate for *M. tuberculosis*, without any statistically significant difference, as compared with combinations B or C. Our findings do not support the assumption that the use of two non-radioactive liquid media is more efficient than that of a liquid plus a solid medium. Statistically significant differences were found when combination A, B or C was compared with the combination of the solid media (combination D) ($p<0.01$), similarly as observed from a comparison of

MGIT, Bactec 12B and solid media by others (Pfyffer et al., 1997).

Table 5.2.2.

Rates of recovery of mycobacteria and *M. tuberculosis* for liquid and solid media in combination.

No. of isolates	Combination A n (%)	Combination B n (%)	Combination C n (%)	Combination D n (%)
Mycobacteria n = 117	109 (93.2)	108 (92.3)	103 (88.0)	85 (72.6)
M. tuberculosis n = 112	104 (92.9)	106 (94.6)	99 (88.4)	84 (75.0)

χ^2 test for differences in recovery of mycobacteria and *M. tuberculosis*: combination A, B, or C vs. D, $p<0.01$, significant.

The mean time to detection of *M. tuberculosis* was 16.5 (2-42), 13.3 (2-33), 24.2 (13-59) and 20.4 (7-53) days for MGIT, MB Redox, LJ and Middlebrook 7H11, respectively. The statistical test revealed statistically significant differences between MGIT, MB Redox, LJ and Middlebrook 7H11 ($p<0.05$). The mean times to detection of *M. tuberculosis* in smear-positive specimens with MGIT and MB Redox were comparable (7.2 versus 6.9 days) (Table 5.2.3). These times are shorter than the previous MGIT reports of 9.0 days, 9.9 days or 15.3 days (Casal et al., 1997; Pfyffer et al., 1997; Rivera et al., 1997), but in our study the number of smear-positive specimens was much lower. The mean times to detection of *M. tuberculosis* in smear-positive specimens were much shorter with MGIT and MB Redox than those for the solid media (Table 5.2.3). The mean time to detection of *M. tuberculosis* in smear-negative specimens was longer with MGIT than with MB Redox (19.1 versus 15.5 days). The time for MGIT accorded well with the reports of 20.3 days by Pfyffer et al.

(1997), and 18.6 days by Rivera et al. (1997), but was inconsistent with the 14.0 days found by Casal et al. (1997). The time for MB Redox (15.5 days) was shorter than those observed by Pfyffer et al. (1997) and Rivera et al. (1997), but was in line with that given by Casal et al. (1997) for MGIT. We have not found any previous data on the recovery times of smear-positive and negative specimens with MB Redox with which to compare our findings. However, our data indicate that the MB Redox method may be much faster for the recovery of *M. tuberculosis* from smear-negative specimens than the MGIT method. An excellent mean time was obtained for smear-negative *M. tuberculosis*-positive specimens on Middlebrook 7H11 slants (21.6 days), this being comparable to that for MGIT (19.1 days) (Table 5.2.3). Both liquid media were significantly faster than LJ for smear-negative *M. tuberculosis*-positive specimens, and MB Redox was also significantly faster than Middlebrook 7H11 (Table 5.2.3).

Table 5.2.3.

Mean time to detection of mycobacteria and *M. tuberculosis* in clinical specimens.

Media	Mycobacteria days (range)	*M. tuberculosis* smear-positive days (range)	*M. tuberculosis* smear-negative days (range)
MGIT	17.0 (2-56)	7.2 (2-10)	19.1 (14-42)
MB Redox	14.7 (2-58)	6.9 (2-11)	15.5 (12-33)
LJ	24.6 (13-59)	20.4 (13-46)	25.8 (16-59)
M-7H11	20.4 (7-53)	17.6 (7-27)	21.6 (14-53)

Analysis of variance, $p<0.001$.

Newman-Keuls test for differences in mean times to detection of mycobacteria: MGIT vs.LJ, $p<0.05$, significant.

Newman-Keuls test for differences in mean times to detection of *M.*

tuberculosis: MGIT vs. MB Redox vs. LJ vs. Middlebrook-7H11, $p<0.05$, significant.

The low number of NTM did not allow an exact statistical comparison of this group. Studies with larger numbers of isolates are needed to determine the reliability of MGIT and MB Redox for the detection of NTM. The contamination rates for MGIT, MB Redox, LJ and Middlebrook 7H11 were 4.3%, 3.9%, 2.1% and 2.7% (Table 5.2.1). Statistical analysis did not reveal any significant difference.

The advantages of the MGIT and MB Redox systems are the rapidity, the high sensitivity, the easy detection of growth, and the lack of need for needles, radioactive materials and specialized instrumentation. However, in 5 MGIT vials without fluorescence, only the observation of non-homogeneous turbidity and small grains revealed the AFB positivity. This could be a source of false-negative reports with the recently-introduced automated MGIT system. The contamination rates of the two liquid media were also acceptable and did not cause any problem. Although the addition of oleic acid-albumin-dextrose-catalase and PANTA to MGIT is an inconvenient extra step, the original lack of the antibiotic mixture in the broth provides a longer shelf-life. Furthermore, no refrigeration is needed during storage, in contrast with the ready-to-use MB Redox with polymyxin B, amphotericin B, carbenicillin, trimethoprim (PACT). The PACT present in MB Redox may also decrease the mycobacterial growth in specimens from sterile body sites, while the inoculation of such specimens into MGIT without the addition of PANTA allows this problem to be avoided. In our experience, the examination of MB Redox for growth positivity is easier because the reading of MGIT specimens under UV light can be eye-stressing. The MGIT system is approximately twice as expensive as MB Redox, but both media remain cost-effective in laboratories that cannot afford the expensive Bactec 460 TB equipment.

In summary, the MGIT and MB Redox systems may be considered viable alternatives to the radiometric Bactec 460 TB system for the diagnosis of tuberculosis.

5.3. Evaluation of the automated MB/BacT system versus the semi-automated Bactec 460 TB system and solid medium:

In accordance with previous reports, the Bactec system provided excellent sensitivity and rapidity in the detection of *M. tuberculosis* (Roberts et al., 1983; Anargyros et al.1990; Abe et al., 1992). Our results also demonstrate that MB/BacT has a sensitivity comparable with that of the radiometric method. However, we found that the semi-automated system was capable of detecting *M. tuberculosis* almost 4 days earlier than MB/BacT. In a previous study, MB/BacT was reported to be similar in sensitivity and rapidity to Bactec 12B for the detection of mycobacteria (Rohner et al., 1997). Unfortunately, in that report did not furnish data on the rates of recovery and the mean times to detection of *M. tuberculosis*. Therefore, we compared our results with the detailed findings of a more recent study (Benjamin et al., 1998). In contrast with our results, that study indicated a higher sensitivity (96%) and a shorter (13.7 days) mean time to detection of *M. tuberculosis* with the MB/BacT system. However, Benjamin et al. (1998) detected only 23 *M. tuberculosis* isolates, while in our study the number of *M. tuberculosis* isolates was much higher, 48. For this reason, the better parameters of their study may only reflect the lower number of isolates identified. Furthermore, Benjamin et al. (1998) used a revised reconstitution fluid for the antibiotic supplement with oleic acid as a substitute for Tween 80 to enhance mycobacterial growth.

In line with both previous studies, the rate of contamination was lower with Bactec 12B than with MB/BacT. The contamination rate of 7.5% for MB/BacT in our study was comparable with the 7.0% reported by

Benjamin et al. (1998) and lower than the 9.4% found by Rohner et al. (1997). These higher rates of contamination with the MB/BacT system are probably a result of the fact that the media are richer in nutrients than the Bactec 12B media. However, it is interesting that the contamination rate published for MB/BacT by Benjamin et al. (1998) did not differ from our finding, although they used a modified antibiotic supplement with vancomycin.

As concerns the instrumentation, it is not irrelevant that the Bactec equipment occupies less space in the laboratory. Unfortunately, positive Bactec 12B vials have to be monitored daily until the GIs have increased, allowing a ZN staining to confirm the growth positivity. Moreover, the needles of the instrument have to be changed and sterilized daily. A considerable amount of technical time is also spent on loading and unloading racks into the Bactec instrument. This obviously eliminates the use of a continuously monitored system.

The MB/BacT system provides a fully automated reading during the incubation period. Once the specimens have been inoculated and the vials have been registered in the instrument, no further handling is required. The automated detection unit provides continous monitoring, an immediate notification of positives and barcode tracking. For these reasons, the workload with MB/BacT is much lower than that with Bactec. Further advantages are that the MB/BacT system has computer-based data management capabilities, there is no need for radioactive substrates and therefore no radioactive waste is created. However, the MB/BacT system has a major drawback: the low capacity of the instrument. While one Bactec instrument allows the processing of 60 vials per hour, an MB/BacT incubator cabinet has a capacity of only 149 new specimens per a month (for an incubation period of 7 weeks). In other words, measurement of the 8000 specimens received annually by our laboratory would require at least four MB/BacT cabinets.

In summary, the MB/BacT system has been shown to be a highly sensitive method for the culturing of clinical specimens for the recovery of *M. tuberculosis*. The continuous monitoring and hands-off operation of the equipment allow the rapid (as compared with the solid medium) detection of a positive culture with minimal technical interventions (as compared with Bactec). However, despite the above-mentioned advantages, we conclude that MB/BacT requires further improvements as regards the time to detection of *M. tuberculosis* and the capacity. In our opinion, because of its capacity, the MB/BacT system in its present form is not a viable alternative to the radiometric system. The number of NTM was too low in this study, and further evaluations with higher numbers of isolates are therefore needed to establish the efficacy of MB/BacT in this respect.

5.4. Evaluation and introduction of the Amplicor polymerase chain reaction assay for the routine direct detection of *M. tuberculosis*:

In recent years, various set-ups for amplification and identification of mycobacterial DNA by the PCR technique have been reported (Eisenach et al., 1990; Glennon et al., 1994; Soini et al., 1994; Zolg and Philippi-Shulz, 1994). However, to date no easy-to-handle test kit has been developed which would allow this method to be established in a routine tuberculosis laboratory. The Amplicor assay is the first PCR test which has been developed in kit form for use in a clinical laboratory for the detection of *M. tuberculosis*. The test is performed directly on processed respiratory specimens without the need for extensive nucleic acid extraction. The assay offers a simple protocol which is compatible with the routine clinical flow of work and can be completed in about 6.5 hours. However, several modifications of normal clinical laboratory procedures need to be made to perform the test successfully. Three separate work areas for the different parts of the test, dedicated pipettes with filtered tips and increased care during pipetting are necessary. Since the

decontamination requires about 2 hours, it is possible to incorporate the assay into the normal work flow with the ability to report results in 24 hours (Somosközi et al., 1997A; Deák et al., 1998).

The published evidence supports the concept that PCR assays provide the ability to detect non-cultivable organisms in patients receiving adequate antituberculosis therapy (Kennedy et al., 1994; Levee G et al., 1994; Somosközi et al., 1995). When culture alone is utilized as the standard method in a comparison study, specimens containing non-cultivable mycobacteria which are positive by the PCR test are initially defined as false-positive specimens. It also noteworthy that culture itself can be false-negative in as many as 29.5% of patients with tuberculosis (Nardell et al., 1995). To avoid misinterpretation of the results, a rigorous discrepancy analysis must be performed on these specimens to determine what proportion of the specimens with initial false-positive results contain non-cultivable organisms that culture cannot detect. The results from this analysis can be used to correct the initial results and to calculate the final performance values. The discrepancy analysis was based on the clinical history of the patients (including the response to therapy) and additional non-study culture and PCR results. As a result of the analysis of specimens with discrepant results, the results on 108 specimens that were missed by culture were considered true-positive, while the remaining 12 specimens were considered false-positive by Amplicor PCR. The positivity of these 108 specimens presumably resulted from *M. tuberculosis* organism that was either non-viable because of the treatment or because of overzealous decontamination. Knowledge of the clinical status of the patient is available to the clinician at the time of the examination, and no significant difficulty should therefore arise in the interpretation of the results of PCR. In the 12 verified false-positive specimens, the cause is unlikely to relate to carry-over contamination, because of the use of uracil-N-glycosylase (AmpErase) (Longo et al., 1990). However, it may result from cross-contamination of another

positive sample (Small et al., 1993).

The initial sensitivity of the Amplicor PCR test as compared with that of culture was 74.5%. Sixty-six percent of the samples missed by Amplicor PCR were low-positive, containing fewer than 10 CFU of *M. tuberculosis* on the parallel LJ slant. This lack of sensitivity for low-positivity specimens was probably due to the uneven distribution of the mycobacteria in the specimens and the low effective inoculum for Amplicor PCR as compared with that for culture. The effective inoculum for Amplicor PCR is 25 μl; thus only 1.6% of the specimen was analysed by PCR, whereas up to 90% was analysed by culture. In line with previous reports, inhibitors were a minor factor in the overall sensitivity of Amplicor PCR (Andersen et al., 1993; Moore et al., 1995). Inhibition of amplification occurred in less than 1% of all samples. The estimation of sensitivity is highly influenced by the percentage of smear-positive samples. One of the best ways to measure the benefit of a PCR test, therefore, is to calculate the increment in sensitivity as compared with the sensitivity of microscopy. In our study, a high increment in the sensitvity was obtained over microscopy by Amplicor PCR (initial sensitivity: 74.5% vs. 23.4%; clinical sensitivity: 80.6% vs. 16.2%). Our results demonstrate that the Amplicor PCR can detect the presence of *M. tuberculosis* in 66.6% of ZN-negative and culture-positive specimens at the time of admission without an overview of the patient records. Discrepancy analysis can improve the sensitivity of PCR in these specimens to 80.6% without a considerable loss of time. The initial specificity of the Amplicor PCR was 92.8%. By using the final and resolved results, the clinical sensitivity, specificity, PPV and NPV were 83.8%, 99.2%, 95.4% and 97%, respectively for Amplicor PCR, 63.5%, 100%, 100% and 93.5%, respectively for culture, and 16.2%, 100%, 100% and 86.3%, respectively for ZN. The low sensitivity achieved for culture is not surprising because only two LJ slants, but not liquid media were used.

We conclude that the present study of a large number of specimens has

demonstrated a high sensitivity (Amplicor vs. culture and smear, $p<0.01$) and an excellent specificity of Amplicor PCR. Our results indicate that the majority of *M. tuberculosis*-positive specimens can be identified rapidly by means of the test. In addition, with a NPV of almost 100%, the assay can rapidly help to exclude tuberculosis from the differential diagnosis. When the specimen is microscopically positive but Amplicor PCR-negative, the test may indicate the presence of NTM. However, since the sensitivity of Amplicor PCR is not equal to that of culture in all instances (the initial and clinical sensitvities of Amplicor PCR are 66.6% and 80.7% for ZN-negative specimens), a culture technique must still be used to detect any missed positive specimens and to provide an isolate for drug susceptibility testing. In a recent multicentre study, involving six laboratories a total of 7194 specimens were evaluated by Amplicor PCR and culture and comparable initial and resolved values were reported for Amplicor PCR and culture (Bennedsen et al., 1996).

In summary, the Amplicor PCR test is a rapid, reliable and valuable adjunct of the routine clinical mycobacteriology laboratory. However, its application requires technical expertise and dedicated laboratory facilities. It is recommended only for laboratories which simultaneously perform culture and microscopy for control of the performance of PCR tests.

6. Concluding Remarks:

The early laboratory diagnosis of tuberculosis still relies on the examination of stained smears. However, the sensitivity of microscopy is not always satisfactory and it is time-consuming. To eliminate these drawbacks, we have developed and constructed a computer-directed automated microscope. To our knowledge, no similar equipment has been applied in mycobacteriology or even in microbiology. Our investigations have proved that the presence of AFB can safely be detected with an automated walk-away microscope in either clinical specimens or liquid media. We have demonstrated that the automated system is more rapid than the manual method and the instrument could always examine the required number of viewfields according to standard recommendations, each of them with the same care. We conclude that, at present, only this equipment affords a possibility for a significant reduction in the chance of false-negative reports of microscopy, because the effects of fatigue during manual microscopy can be excluded, the standardized method of microscopic examination can always be followed and the examination can easily be quality-controlled. Moreover, our results have demonstrated that application of the automated microscope developed by our laboratory is less labour-intensive, and hence may free laboratory staff for other duties and may permit rapid and reliable screening of the population at risk in large numbers.

The definitive diagnosis of tuberculosis depends on the isolation and identification of the aetiologic agent, *M. tuberculosis*. The use of gold standard laboratory technologies is a must for the rapid and accurate microbiological diagnosis of the disease. Currently, the gold standard for *M. tuberculsosis* testing methodologies combines radiometric growth detection by the Bactec 460 TB system and cultivation on solid media. However, the Bactec system is still labour-intensive and the high cost of acquisition and the accumulation of radioactive waste are serious

drawbacks. To circumvent these limitations, two new, non-radiometric broth-based methods were recently introduced, involving MGIT and MB Redox media. Although preliminary studies have demonstrated that MGIT is of promise, those studies were carried out with a limited number of specimens or MGIT was compared with a single medium (only with Bactec 12B or only with LJ). Furthermore, the results on smear-positive and smear-negative specimens remained unknown (Kodsi et al., 1994; Neumann et al., 1995; Palaci et al., 1996; Casal et al., 1997; Rivera et al., 1997). This parameter is very important, since the recovery and the time to detection of AFB are greatly influenced by the number of organisms present. Currently, only one report of a multicentre study provides any reliable data on MGIT and only one congress abstract on MB Redox exists (Naumann et al., 1996; Pfyffer et al., 1997). Besides providing more detailed results concerning the evaluation of the two new media in comparison with previous reports, our study is the first and, for the time being, the only one in which the two non-radiometric methods have been compared with each other. The information summarized in our study is crucial for laboratories which are considering replacing the radioactive method, and especially for those laboratories that are planning to introduce broth-based cultivation, but cannot afford expensive instrumentation, as is the case for laboratories in Hungary.

Automation of the cultivation process is high on the list of desires of laboratories dealing with a large daily specimen load. For many years, the only mycobacterial culture system that offered any automation was the radiometric Bactec 460 TB system. In the Bactec system, specimens are read twice weekly for the first 2 weeks and once a week thereafter for a total of 6 weeks. The disadvantages of the system include the manual loading of culture bottles, the need to establish a reading schedule and the problems involving the disposal of radioactive waste. The recently developed MB/BacT system provides a fully automated reading during the incubation period. Once the specimens have been inoculated and the

vials have been registered in the instrument, no further handling is required. The automated detection unit provides continuous monitoring and an immediate notification of positives. For these reasons, the workload with MB/BacT is much lower than that with Bactec. Similarly to the MGIT and MB Redox systems, there is no need for radioactive substrates and therefore no radioactive waste is created. However, at present only two reports have been published on MB/BacT, and there is therefore not enough experience with this system. In one of the previous studies, MB/BacT was found to be similar in sensitivity and rapidity to Bactec 12B for the detection of mycobacteria (for *M. tuberculosis* and NTM together), but unfortunately, no data were reported on the rates of recovery and the mean times to detection of *M. tuberculosis* (Rohner et al., 1997). Although these data were discussed in the more recent study of Benjamin et al. (1998), they detected only 23 *M. tuberculosis* isolates, which is too low for reliable conclusions. In order to supply the information not provided by the previous two studies, we conducted a clinical trial for a reliable evaluation of the MB/BacT system. Our results demonstrated that the MB/BacT system was capable of detecting *M. tuberculosis* only almost 4 days later than the Bactec system. The contamination rate for MB/BacT was also higher (7.5%) than that with the Bactec 12B (4.0%). However, in our experience, the major drawback of the MB/BacT system is the low capacity of the instrument. The measurement of the 8000 specimens received annually by our laboratory would require at least four MB/BacT cabinets, which is very expensive even for laboratories in industrialized countries.

Between 1996 and 1998, our laboratory introduced four broth-based cultivation methods in Hungary: the semi-automated Bactec 460TB system, the manual MGIT and MB Redox systems and the fully-automated MB/BacT system. The conclusion drawn from investigations with these systems is that, apart from its disadvantages, the Bactec 460 TB system is still the gold standard of broth-based cultivation. Since the

sensitivity and the rapidity of cultivation are significantly higher when liquid and solid media are used in combination, the wider introduction of liquid media in Hungary should be a must. Our results showed that MGIT and MB Redox can be viable alternatives to Bactec 460 TB. Although the MGIT system is approximately twice as expensive as MB Redox, both media remain cost-effective in laboratories that cannot afford the expensive Bactec 460 TB instrument. In consequence of its lower price for Hungarian laboratories, application of the MB Redox system is more acceptable. The MB/BacT system provides a fully automated, walk-away cultivation process, but because of the above-mentioned serious drawbacks, we conclude that further improvements are required before its introduction into the routine work.

The application of nucleic acid amplification techniques for the direct detection of *M. tuberculosis* has offered considerable changes in mycobacteriology. Currently, most experience is available from the PCR in this respect. Several authors have published different „home-made" PCR procedures capable of detecting *M. tuberculosis* in clinical specimens (Brisson-Noel et al., 1989; Eisenach et al., 1990; Soini et al., 1994). However, the results from a recent study in which the performances of seven clinical laboratories were compared highlighted an unexpectedly high variation in sensitivity that can occur when PCR is performed by using different tests and non-standardized reagents and protocols (Noordhoek et al., 1993). Furthermore, it is very important that the application of PCR in the clinical diagnosis of tuberculosis necessitates the distinction of the „analytical" and „clinical" sensitivity and specificity of the test. On an analytical level, PCR provides a sensitivity and a specificity comparable those of with culture in solutions of predetermined composition. However, as our results proved, under clinical conditions the sensitivity and specificity are greatly influenced by the number, the inhomogeneity and the viability of bacteria in samples. Moreover, it has become clear that the use of culture as a gold standard to determine

these parameters is insufficient, because patients with tuberculosis can give negative cultures. Discrepancies can be resolved by utilizing the clinical data on the patients. In the recently commercially developed Amplicor PCR assay, the reagents are standardized and quality-controlled to ensure performance and for this reason it might overcome the above-mentioned difficulties. However, for any assay to be useful in a clinical diagnostic setting, a careful validation is needed for the local clinical conditions because sensitivity is also influenced by factors determined by the patient population under investigation (mainly by the rate of smear-positive samples) (Fodor, 1996). Determination of the local initial and clinical sensitivity, specificity, PPV and NPV of a PCR test is therefore indispensable. The results of our study, based on a large number of examined specimens, represent the Hungarian parameters of the standardized Amplicor PCR test. These results are essential to allow Hungarian clinicians to interpret PCR results in accordance with local conditions.

We conclude that this first Hungarian study on the application of PCR for the direct detection of *M. tuberculosis* has demonstrated a high sensitivity (Amplicor vs. culture and smear, $p<0.01$) and an excellent specificity of Amplicor PCR. Our results indicate that the majority of *M. tuberculosis*-positive specimens can be identified rapidly with the test and, because of the high NPV, the Amplicor PCR test can help to exclude tuberculosis from the differential diagnosis in 24 hours. We have also demonstrated that the Amplicor PCR can detect the presence of *M. tuberculosis* in 66.6% of ZN-negative and culture-positive specimens at the time of admission without an overview of the patient records. Discrepancy analysis can improve the sensitivity of PCR in these specimens to 80.6% without a considerable loss of time, because the knowledge of the clinical status of the patients is available to the clinician at the time of the examination.

It is noteworthy that, although the introduction of PCR can lead to improved clinical management, the results must be interpreted with extreme caution, and this procedure requires technical expertise and dedicated laboratory facilities. It is recommended only for laboratories which simultaneously perform culture and microscopy for control of the performance of PCR tests.

7. Acknowledgements:

The author wishes to express his thanks to:

Professor Erzsébet Nagy, his supervisor, for her excellent support and encouragement;

Dr. Judit Deák, his supervisor, for inspiring discussions and her good advice;

Professor Pál Magyar, his supervisor, for his continuous support and encouragement, and for providing the facilities for scientific research;

Professor Imre Hutás, for stimulating discussions and his great personal support;

Dr. Lajos Nagy, for introducing him to the field of mycobacteriology;

Dr. Sabine Rüsch-Gerdes, Professor Joachim Müller-Quernheim and Dr. Gernot Zissel, his tutors, for sharing their great knowledge and introducing him into the field of research;

Donát Kiss and Dr. Nóra Czoboly for the engineering and construction of the automated microscope, and for their encouraging attitude;

Zsuzsanna Szilágyi, Katalin Gulyás, Klára Muránszky, Doris Bubritzki and Nicolle Husmann for their excellent technical assistance.

8. References:

Abe C, Hosojima S, Fukasawa Y. Comparison of MB-Check, BACTEC and egg-based media for recovery of mycobacteria. J Clin Microbiol 1992; 30:878-881.

Anargyros P, Astill DSJ, Lim ISL. Comparison of improved BACTEC and Löwenstein-Jensen media for culture of mycobacteria from clinical specimens. J Clin Microbiol 1990; 28:1288-1291.

Andersen AB, Thybo S, Godfrey-Faussett P, Stoker NG. Polymerase chain reaction for detection of *Mycobacterium tuberculosis* in sputum. Eur J Clin Microb Infect Dis 1993; 12:922-927.

A Pulmonológiai Intézetek 1990-1995. évi epidemiológiai és működési adatai. OKTPI, 1991-1996.

Casal M., Gutierrez J, Vaquero M. Comparative evaluation of the Mycobacteria Growth Indicator Tube with the BACTEC 460 TB system and Löwenstein-Jensen medium for isolation of mycobacteria from clinical specimens. Int J Tuberc Lung Dis 1997; 1:81-84.

Cohn ML, Waggoner RF, McClatchy JK. The 7H11 medium for the culture of mycobacteria. Am Rev Respir Dis 1968;98:295-296.

Benjamin WH, Waites KB, Beverly A, Gibbs L, Waller M, Nix S, Moser SA, Willert M. Comparison of the MB/BacT system with a revised antibiotic supplement kit to the Bactec 460 system for detection of mycobacteria in clinical specimens. J Clin Microbiol 1998; 36:3234-3238.

Bennedsen J, Thomsen OV, Pfyffer GE, Funke G, Feldmann K, Beneke A, Jenkins A, Hegginbothom M, Fahr A, Hengstler M, Cleator G, Klapper

P, Wilkins EGL. Utility of PCR in diagnosing pulmonary tuberculosis. J Clin Microbiol 1996; 34:1407-1411.

Böddinghaus B, Rogall T, Flohr T, Blöcker, H, Böttger EC. Detection and identification of mycobacteria by amplification of rRNA. J Clin Microbiol 1990; 28:1751-1759.

Brisson-Noel A, Gicquel B, Lecossier D, Lévy-Frébault V, Nassif X, Hance AJ. Rapid diagnosis of tuberculosis by amplification of mycobacterial DNA in clinical samples. Lancet 1989; 4:1069-1071.

Deák J, Mislóczky M, Somosközi Á, Nagy E, Ferencz A: A Mycobactertium tuberculosis fertőzések Magyarországon alkalmazott modern, mikrobiológiai diagnosztikai módszerei. Infektológia és Klinikai Mikrobiológia. 1998; 3:114-123.

Eisenach KD, Cave MD, Bates JH, Crawford JT. Polymerase chain reaction amplification of a repetitive DNA sequence specific for *Mycobacterium tuberculosis*. J Infect Dis 1990; 161: 977-981.

Fodor T. Bulk staining of sputum by Ziehl-Neelsen method. Tubercle 1984; 65:123.

Fodor T.: Detection of mycobacteria in sputum smears prepared by cytocentrifugation and sedimentation. Tubercle 1995; 76:273-274.

Fodor T. Hogyan használhatók a gümőbaktériumok kimutatására szolgáló nukleinsavpróbák? Medicina Thoracalis 1996; 49:445-446.

Gergely P. A tuberkulózis immunológiája. Medicina Thoracalis 1998; 51:185-188.

Glennon M, Smith T, Cormican M, Noone D, Barry T, Maher M, Dawson M, Gilmartin JJ, Gannon F. The ribosomal intergenic spacer-region: a target for the PCR based diagnosis of tuberculosis. Tuber Lung Dis 1994; 75:353-360.

Gordin F, Slutkin G. The validity of acid-fast smears in the diagnosis of pulmonary tuberculosis. Arch Pathol Lab Med 1990; 114:1025-1027.

Hanna BA. Diagnosis of tuberculosis by microbiologic techniques. In: Rom WN and Garay SM ed. Tuberculosis. Boston, Little Brown & Co. 1996: pp 149-159.

Heifets LB, Good RC. Current laboratory methods for the diagnosis of tuberculosis. In: Bloom BR. ed. Tuberculosis: pathogenesis, protection, and control. Washington, DC; ASM Press 1994: pp 85-110.

Hutás I. A tüdőgümőkór. In: Magyar P, Hutás I, Vastag E ed. Pulmonológia. Budapest; Medicina 1997: pp 312-337.

Kennedy N, Gillespie SH, Saruni AOS, Kisyombe G, McNerney R, Ngowi FI, Wilson S. Polymerase chain reaction for assessing treatment response in patients with pulmonary tuberculosis. J Infect Dis 1994; 170:713-716.

Kent PT, Kubica GP. Public health mycobacteriology. A guide for the level III laboratory. Atlanta, Georgia; Centers for Disease Control, 1985.

Kim TC, Blackman RS, Heatwole AM. Acid-fast bacilli in sputum smears of patients with tuberculosis: prevalence and significance of negative smears pretreatment and positive smears post-treatment. Am Rev Respir Dis 1984; 129:264-270.

Kodsi SE, Hagemann PA, Douglass JD, Stitt DT. Comparison of the BBL

MGIT Mycobacteria Growth Indicator Tube with the Bactec TB system using clinical specimens. Abstr. D-55, p.155. In: Program and Abstracts of the 34th Interscience Conference on Antimicrobial Agents and Chemotherapy, American Society for Microbiology 1994.

Kubica GP, Dye WE, Cohn ML, Middlebrook G. Sputum digestion and decontamination with N-acetyl-L-cysteine-sodium hydroxide for culture of mycobacteria. Am Rev Respir Dis 1963; 87:775-779.

Levee G, Glaziou P, Gicquel B, Chanteau S. Follow-up of tuberculosis patients undergoing standard anti-tuberulosis chemotherapy by using a polymerase chain reaction. Res Microbiol 1994; 145:5-8.

Longo MC, Berninger MS, Hartley JL. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. Gene 1990; 93:125-128.

Middlebrook G, Cohn ML. Bacteriology of tuberculosis: laboratory methods. Am J Public Health 1958;48:844-853.

Middlebrook G, Reggiardo Z, Tigertt WD. Automatable radiometric detection of growth of *M. tuberculosis* in selective media. Am Rev Respir Dis 1977; 115:1066-1069.

Moore DF, Curry JI. Detection and identification of *Mycobacterium tuberculosis* directly from sputum sediments by Amplicor PCR. J Clin Microbiol 1995; 33:2686-2691.

Morgan MA, Horstmeier CD, DeYoung DR, Roberts GD. Comparison of a radiometric method (Bactec) and conventional culture media for recovery of mycobacteria from smear-negative specimens. J Clin Microbiol 1983; 18:384-388.

Mullis KB, Faloona FA. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol 1987; 155:335-350.

Nardell E, Bautolte J, Shampra Pai S, Etkind S. Tuberculosis without culture confirmation. Am Rev Respir Dis 1995; 151:A336.

Naumann L, Lang E, Pausch G, Oros H, Vilsmeier S, Haber B, Reischl U, Linde HJ. A new rapid cultural non-radiometric method for the detection of mycobacteria. Abstr. P-93, p.92. In: Abstracts of the 17th Annual Meeting of the European Society for Mycobacteriology 1996.

Neumann MA, Rogers J, Williams R, Maxwell S. Comparison of the new Mycobacteria Growth Indicator Tube (MGIT) with the Bactec 460 TB system for the rapid detection of mycobacteria from clinical specimens. Abstr. C-138, p.24. In: Abstracts of the 95th General Meeting of the American Society for Microbiology 1995.

Nolte FS, Metchcock B. Mycobacterium. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH ed. Manual of clinical microbiology, 6th ed. Washington, DC; ASM Press 1995: pp 400-437.

Noordhoek GT, van Embden JDA, Kolk AHJ. Questionable reliability of the polymerase chain reaction in the detection of *M. tuberculosis*. N Engl J Med 1993; 329:2036.

Palaci M, Ueki SYM, Sato DN, da Silva Telles MA, Curcio M, Silva EAM. Evaluation of Mycobacteria Growth Indicator Tube for recovery and drug susceptibility testing of *Mycobacterium tuberculosis* isolates from respiratory specimens. J Clin Microbiol 1996; 34:762-764.

Pataki G. Előzetes jelentés a Pulmonológiai Intézetek 1998. évi epidemiológiai és működési adataiból. 1999.március 18.

Persing DH. In vitro nucleic acid amplification techniques. In: Persing DH, Smith TF, Tenover FC, White TJ. ed. Diagnostic Molecular Microbiology; Principles and Applications. Washington, DC; ASM Press 1993: pp 51-87.

Pfyffer GE, Kissling P, Jahn EMI, Welscher H-M, Salfinger M, Weber R. Diagnostic performance of Amplified Mycobacterium Tuberculosis Direct Test with cerebrospinal fluid, other nonrespiratory, and respiratory specimens. J Clin Microbiol 1996; 34:834-841.

Pfyffer GE, Welscher HM, Kissling P, Cieslak C, Casal M, Gutierrez J, Rüsch-Gerdes S. Comparison of the Mycobacteria Growth Indicator Tube (MGIT) with radiometric and solid media for recovery of acid-fast bacilli. J Clin Microbiol 1997; 5:364-368.

Reischl U, Lehn N, Wolf H, Naumann L. Clinical evaluation of the automated Cobas Amplicor MTB assay for testing respiratory and nonrespiratory specimens. J Clin Microbiol 1998; 36:2853-2860.

Rivera AB, Tupasi TE, Grimaldo ER, Cardano RC, Co VM. Rapid and improved recovery rate of *M. tuberculosis* in Mycobacteria Growth Indicator Tube combined with solid Löwenstein-Jensen medium. Int J Tuberc Lung Dis 1997; 5:454-459.

Roberts GD, Goodman NL, Heifets L, Larsh HW, Lindner TH, McClatchy JK, McGinnis MR, Siddiqi SH, Wright P. Evaluation of the Bactec radiometric method for recovery of mycobacteria and drug susceptibility testing of *Mycobacterium tuberculosis* from acid-fast smear-positive specimens. J Clin Microbiol 1983; 18:689-696.

Roberts GD, Koneman EW, Kim YK. Mycobacterium. In: Balows A, Hausler WJ, Hermann KL, Isenberg HD, Shadomy HJ ed. Manual of Clinical Microbiolgy, 5th ed. Washington, DC; ASM Press 1991: pp 304-339.

Rohner P, Ninet B, Metral C, Emler S, Auckenthaler R. Evaluation of the MB/BacT system and comparison to the Bactec 460 system and solid media for isolation of mycobacteria from clinical specimens. J Clin Microbiol 1997; 35:3127-3131.

Saceanu CA, Pfeiffer NC, McLean T. Evaluation of sputum smears concentrated by cytocentrifugation for detection of acid-fast bacilli. J Clin Microbiol 1993; 31:2371.

Saiki RF, Scharf S, Faloona FA, Mullis KB, Horn GT, Erlich HA, Arnheim N. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia. Science 1985; 230:1350-1354.

Salfinger M, Pfyffer GE. The new diagnostic mycobacteriology laboratory. Eur J Clin Microbiol Infect Dis 1994; 13:961-979.

Schluger NW, Rom WN. The host immune response to tuberculosis. Am J Respir Crit Care Med 1998; 157:679-91.

Shawar RM, El Zaatari FAK, Nataraj A, Clarridge JE. Detection of *Mycobacterium tuberculosis* in clinical samples by two-step polymerase chain reaction and non-isotopic hybridization methods. J Clin Microbiol 1993; 31:61-65.

Small PM, McLenny NB, Singh SP, GK Schoolnik, Tompkins LS, Mickelsen PA. Molecular strain typing of *Mycobacterium tuberculosis* to

confirm cross-contamination in the mycobacteriology laboratory and modification of procedures to minimize occurrence of false-positive cultures. J Clin Microbiol 1993;31:1677-1682.

Smithwick RW. Laboratory manual for acid-fast microscopy. Atlanta, Georgia; Centers for Disease Control, 1976.

Smithwick RW. The working mycobacteriology laboratory. In: Friedman LN. ed. Tuberculosis: current concepts and treatment. Washington, DC; CRC Press 1994: pp 71-80.

Snider DE, Raviglione M, Kochi A. Global burden of tuberculosis. In: Bloom BR. ed. Tuberculosis: pathogenesis, protection, and control. Washington, DC; ASM Press 1994: pp 3-11.

Soini H, Böttger EC, Viljanen MK. Identification of mycobacteria by PCR-based sequence determination of the 32 kilodalton protein gene. J Clin Microbiol 1994; 32:2944-2947.

Sommers HM, McClatchy JK, Monella JA. Laboratory diagnosis of the Mycobacterioses. In: Sommers HM, McClatchy JK ed. Cumitech 16. Washington, DC; ASM Press 1983: pp 1-16.

Strumpf IJ, Tsang AY, Sayre JW. Reevaluation of sputum staining for the diagnosis of pulmonary tuberculosis. Am Rev Respir Dis 1979; 119:599-602.

Styrt BA, Shinnick TM, Ridderhof JC, Crawford JT, Tenover FC. Turnaround times for mycobacterial cultures. J Clin Microbiol 1997; 35:1041-1042.0

Somosközi Á, Győri Zs, Czoboly N, Magyar P. Application of a computer-

directed automated microscope in mycobacteriology. Int J Tuberc Lung Dis 1999A; 4:354-357.

Somoskövi Á, Hutás I, Vajda E, Deák J: A Mycobacterium tuberculosis laboratóriumi diagnosztikájának újabb lehetőségei. Háziorvosi Továbbképző Szemle 1997A; 2:72-74.

Somoskövi Á, Magyar P: Mycobacterium tuberculosis complex kimutatása Accuprobe nukleinsav hibridizációs próba segítségével Bactec 12B folyékony táptalajból. Medicina Thoracalis 1997B; 50:9-13.

Somoskövi Á, Magyar P. Comparison of the Mycobacteria Growth Indicator Tube with MB Redox, Löwenstein-Jensen and Middlebrook 7H11 media for recovery of mycobacteria in clinical specimens. J Clin Microbiol 1999B. (Accepted for publication)

Somoskövi Á, Tolnay E, Vajda E, Zsiray M, Tarján E, Magyar P: Mycobacterium tuberculosis laboratóriumi gyorsdiagnosztikája polimeráz láncreakció segítségével. Medicina Thoracalis 1995; 48:3-9.

Somoskövi Á, Zissel G, Zipfel P, Ziegenhagen M, Klaucke J, Haas H, Schlaak M, Müller-Quernheim J: Different cytokine patterns correlate with the extent of the disease in pulmonary tuberculosis. European Cytokine Network 1999C. (Accepted for publication)

Tenover FC, Crawford JT, Heubner LJ, Geiter LJ, Horsburgh CR, Good RC. The resurgence of tuberculosis: is your laboratory ready? J Clin Microbiol 1993; 35:767-770.

Tevere VJ, Hewitt PL, Dare A, Hocknell P, Keen A, Spadoro JP, Young KKY. Detection of *Mycobacterium tuberculosis* by PCR amplification with pan-mycobacterium primers and hybridization to an *M. tuberculosis*-

specific probe. J Clin Microbiol 1996; 34:918-923.

WHO Fact Sheet No.: 93, Geneva, WHO Press Office, October 1995.

Zolg J and Philippi-Shulz S. The superoxide dismutase gene, a target for detection and identification of mycobacteria by PCR. J Clin Microbiol 1994; 32:2801-2812.