IIRIS RAJALAHTI

A Nucleic Acid Amplification Assay in the Diagnosis and Management of Tuberculosis in a Low-incidence Area

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ACADEMIC DISSERTATION
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University of Tampere, Medical School
Tampere University Hospital, Department of Respiratory Diseases and Centre for Laboratory Medicine
Finland

Supervised by
Docent Markku M. Nieminen
University of Tampere

Reviewed by
Docent Paula Maasilta
University of Helsinki
Docent Hanna Soini
University of Turku

Distribution
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33014 University of Tampere
Finland

Tel. +358 3 3551 6055
Fax +358 3 3551 7685
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Abstract

Background: Smear is insensitive and culture is slow, but both methods are essential in tuberculosis (TB) diagnostics. The nucleic acid amplification (NAA) assay is sensitive and rapid, but its role as an additive test in the diagnosis of TB has remained undefined.

Aims: The objective was to estimate the specimen- and patient-based performance of the NAA assay in detecting *Mycobacterium tuberculosis (M. tuberculosis)* complex from sputum specimens, and to assess its usefulness in monitoring treatment response of pulmonary TB patients. In addition, the cost-effectiveness of the NAA assay and its impact on clinical TB practice was evaluated.

Subjects and methods: The study population, altogether 386 subjects and 34 controls, included 327 patients with suspicion of TB, 44 patients with past TB and 15 patients receiving chemotherapy for TB. They were tested by smear, culture and NAA tests. Both laboratory and patient records were reviewed retrospectively. Additionally, a decision tree model was used to evaluate the cost-effectiveness of the NAA assay in diagnosing pulmonary TB.

Results: The overall sensitivity of the NAA assay in detecting M. tuberculosis complex from sputum specimens was 83 % compared to culture; the specificity, PPV and NPV were 99 %, 97 % and 95 %, respectively. In patient-based evaluation the sensitivity increased to 90 % when three specimens per patient were tested. Further, the sensitivity was 100 % for smear-positive and 75 % for smear-negative patients. No false positive NAA results were detected in patients who had residual lung lesions due to previous TB. NAA test results proved inconsistent in TB patients during chemotherapy and no clinically significant difference between the two different types of NAA assay was found. Routine testing of all TB suspects by the NAA assay was not cost-effective, whereas testing of smear-positive patients was less costly and resulted in more appropriate treatment decisions. However, in clinical evaluation the median NAA test result delay was one week, and NAA testing was of value in only part of the smear-positive cases.

Conclusions: The NAA assay is recommended for use in smear-positive patients with TB suspicion, particularly when distinguishing TB from nontuberculous mycobacteria (NTM) or other disease is difficult. It may also be applied to smear-negative patients with high clinical suspicion of TB, and testing of multiple specimens is recommended. A positive NAA test result is indicative of active TB, but in smear-negative patients TB cannot be excluded by negative NAA results. Moreover, qualitative NAA tests were not found useful in monitoring response to TB treatment. Finally, centralizing of NAA testing is recommended in a low-incidence area.

Tiivistelmä

Tausta: Tuberkuloosin toteaminen on usein vaikeaa tuberkuloosibakteerin hidaskasvuisuuden vuoksi. Diagnostiikka on vuosikymmenien ajan perustunut perinteisiin värjäys- ja viljelymenetelmiin. Värjäystestin avulla arvioidaan potilaan tartuttavuutta ja viljelystä voidaan edetä lajinmääritys- ja lääkeherkkyystutkimuksiin sekä epidemiologisiin selvityksiin. Värjäys on epäherkkä ja viljelyn ongelmana on hitaus, mutta korvaavia testejä ei toistaiseksi ole pystytty kehittämään. Tuberkuloosibakteerin nukleiinihapon monistustesti (NAA-testi) on nopea ja herkkä, mutta sen käyttökelpoisuus ja asema perinteisten testien lisänä tuberkuloosin pikadiagnostiikassa on selkiintymätön.

Tavoitteet: Tutkimuksen tarkoituksena oli määrittää kliinisistä yskösnäytteistä NAA-testin herkkyys ja tarkkuus värjäys- ja viljelytesteihin verrattuna sekä arvioida testin kustannustehokkuutta keuhkotuberkuloosin diagnostiikassa. Lisäksi tutkittiin NAA-testin käyttökelpoisuutta keuhkotuberkuloosia sairastavien potilaiden hoitovasteen seurannassa ja selvitettiin testistä saatavaa hyötyä käytännön potilastyössä.

Aineisto ja menetelmät: Värjäys-, viljely- ja NAA-testit tehtiin 386 tutkittavalle ja 34 kontrollihenkilölle. Tutkituista 327:lla oli alkutilanteessa epäily tuberkuloosista (72:lla oli TB), 44 oli aikaisemmin sairastanut tuberkuloosin ja hoidon seurantaryhmässä oli 15 potilasta. Tutkimusaineisto kerättiin sairauskertomuksista ja laboratoriotulosteista. NAA-testin kustannustehokkuutta arvioitiin päätösvuokaavion avulla.

Tulokset: NAA-testin sensitiivisyys ja spesifisyys yskösnäytteissä oli 83 % ja 99 % viljelyyn verrattuna. Potilaittain määritettynä testin sensitiivisyys oli 90 %, värjäyspositiivisilla potilailla se oli 100 % ja värjäysnegatiivisilla 75 %. NAA-testillä ei todettu vääriä positiivisia tuloksia henkilöillä, joilla oli arpia keuhkojen röntgenkuvassa sairastetun tuberkuloosin jäljiltä. Hoidon aikana NAA-testin tulokset olivat potilaiden kliiniseen ja mikrobiologiseen tilanteeseen nähden epäjohdonmukaisia, eikä kahden erityyppisen geenitestin välillä todettu merkittävää kliinistä eroa. Rutiininomainen NAA-testin käyttö tuberkuloosiepäilyn yhteydessä ei ollut kustannustehokasta, mutta värjäyspositiivisten potilaiden lisätestaus tuli varhaishoidon kustannukset huomioiden halvemmaksi kuin yksinomainen perinteisten testien käyttö ja johti useammin oikeaan hoitopäätökseen. Kliinisessä potilastyössä testin tulos saatiin keskimäärin 7 päivässä ja testauksesta oli hyötyä vain osalle värjäyspositiivisista potilastapauksista.

Johtopäätökset: NAA-testausta suositellaan värjäyspositiivisille potilaille, kun kliinisen tilanteen perusteella keuhkotuberkuloosin erottaminen ei-tuberkuloottisen mykobakteerin aiheuttamasta taudista tai muusta sairaudesta on ongelmallista. Värjäysnegatiivisia potilaita voidaan tutkia NAA-testillä, mikäli on vahva epäily keuhkotuberkuloosista, tällöin suositellaan usean näytteen testausta. Positiivinen NAA-testitulos on ensisijaisesti osoitus aktiivista taudista, mutta negatiivinen testitulos värjäysnegatiivisista näytteistä ei sulje pois TB:n mahdollisuutta. Riittävän näytemäärän takaamiseksi ja tulospalvelun parantamiseksi NAA-testien tekoa suositellaan keskitettäväksi valtakunnalliseen laboratorioon matalan tuberkuloosi-ilmaantuvuuden alueella.

Contents

Abbreviations

AFB	acid fast bacteria
AIDS	acquired immunodeficiency syndrome
AMTD	Amplified Mycobacterium Tuberculosis Direct test
BCG	Bacillus Calmette-Guérin
CDC	Centers of Disease Control and Prevention
CFU	colony forming units
CI	confidence interval
DNA	deoxyribonucleic acid
EMB	ethambutol
FDA	Food and Drug Administration
HIV	human immunodeficiency virus
INH	isoniazid
LCx	ligase chain reaction
LTBI	latent tuberculosis infection
MDR	multi-drug resistant
MGIT	Mycobacterium Growth Indicator Tube
MTB	Mycobacterium Tuberculosis
NAA	nucleic acid amplification
NALC	N-acetyl-L-cysteine
NaOH	sodium hydroxide
NPV	negative predictive value
NTM	nontuberculous mycobacteria
PAS	para-aminosalicylic acid
PCR	polymerase chain reaction
PPV	positive predictive value
PZA	pyrazinamid
RIF	rifampin
RLU	relative light unit
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SDA	strand displacement amplification
Se	sensitivity
SM	streptomycin
Sp	specificity
TB	tuberculosis
TMA	transcription mediated amplification
WHO	World Health Organization

List of original communications

This thesis is based on the following original communications, which are referred to in the text by their Roman numerals (I-V).

I Rajalahti I, Vuorinen P, Nieminen MM, Miettinen A (1998): Detection of *Mycobacterium tuberculosis* complex in sputum specimens by the automated Roche Cobas Amplicor Mycobacterium Tuberculosis test. J Clin Microbiol 36:975-978.

II Rajalahti I, Vuorinen P, Järvenpää R, Nieminen MM (2003): *Mycobacterium tuberculosis* complex is not detected by DNA amplification assay in sputum specimens of patients with lung scars due to past pulmonary tuberculosis. Int J Tuberc Lung Dis 7:190-193.

III Rajalahti I, Vuorinen P, Liippo K, Nieminen MM, Miettinen A (2001): Evaluation of commercial DNA and rRNA amplification assays for assessment of treatment outcome in pulmonary tuberculosis patients. Eur J Clin Microbiol Infect Dis 20:746-750.

IV Rajalahti I, Ruokonen E-L, Kotomäki T, Sintonen H, Nieminen MM (2004): Economic evaluation of the use of PCR assay in diagnosing pulmonary TB in a low-incidence area. Eur Respir J 23:446-451.

V Rajalahti I, Luukkaala T, Vuento R, Nieminen MM (2006): The role of PCR testing in management of patients with suspicion of TB in a low-incidence area (submitted).

Introduction

Tuberculosis (TB) is caused by an acid-fast slow-growing organism, *Mycobacterium tuberculosis* (*M. tuberculosis*). While TB-HIV co-infection and multi-drug resistant TB are major challenges globally (Dye et al. 2005), industrialized countries with low TB incidence are also facing the reality of decreasing clinical skills and experience in TB control. WHO launched the new Stop TB Strategy in 2006, which among with other targets further emphasizes the importance of rapid case detection and development of new diagnostic tools (Raviglione and Uplekar 2006). At its shortest drug-sensitive TB is treated with multiple drugs for 6 months. Infectious patients are moreover treated in isolation for the first two weeks. Therefore, from both the individual and epidemiological point of view, rapid and correct diagnosis is essential.

Conventional methods have remained the cornerstone in TB diagnostics. Rapid detection of *M. tuberculosis* from clinical specimens, however, is problematic because smear is insensitive and non-specific. Besides TB, a positive smear result may also indicate nontuberculous mycobacteria (NTM). Culture is currently the gold standard, but the result takes 2-6 weeks. Despite weaknesses, smear is essential in determining infectiousness of the patient, and culturing of the strains is needed for species identification, drug susceptibility testing and genotyping of the strains. But, we are furthermore lacking a rapid diagnostic tool to detect TB.

Since the invention of polymerase chain reaction (PCR) (Saiki et al. 1985, Mullis et al. 1986) molecular techniques have achieved substantial progress, and various nucleic acid amplification (NAA) based assays have been brought onto the market. However, the cost and need for high quality laboratories and technical time impair their usefulness. Moreover, the sensitivity of NAA tests is not optimal in paucibacillary specimens. After extensive research work it has become evident that NAA tests cannot replace the conventional methods, but can be used as complementary tests. Their exact role in the diagnosis of TB, however, has remained undefined.

If a new test is to be implemented, several factors should be considered. Firstly, the test has to be competent in terms of laboratory performance, and secondly, the new test should offer additional benefit compared to former tests. Finally, in the optimal situation the new test should be cost-effective and improve patient care. The aim of this study was to evaluate the performance of the commercial NAA test in detecting *M. tuberculosis* complex from clinical sputum specimens. In addition, the cost-effectiveness of the test and its usefulness in the clinical practice as well as impact on patient management was assessed. All the evaluations were made from the perspective of a low TB incidence area.

Review of the literature

1. Tuberculosis

1.1. Long history shortly

Tuberculosis (TB) is believed to be one of the oldest human diseases. Studies of skeletal remains have revealed that tuberculosis has existed for thousands of years (Roberts and Buikstra 2003, pp. 4-17, Zink et al. 2003, Taylor et al. 2005). Eventually in 1882 Robert Koch first described the tubercle bacillus, the etiology of the white plague (Koch 1882). At that time tuberculosis was the main cause of death in most European countries. Koch´s discovery was a breakthrough leading to other important findings, which contributed to the fight against tuberculosis. He developed staining and culture methods and segregated an extract, tuberculin, which was further elaborated to be used as a skin test to detect tuberculosis infection (Pirquet 1907, Grange 2003, pp. 95-96). Koch was awarded the Nobel Prize in medicine and physiology for his work on TB in 1905. At the beginning of the twentieth century the Bacillus Calmette-Guérin (BCG) vaccine was obtained from an isolated attenuated *Mycobacterium bovis* strain, and was first administered to a human as an oral vaccine in 1921 (Clements 2003, p. 46). The value of BCG vaccination in past decades has been demonstrated especially in preventing serious forms of tuberculosis in young children (Tala et al. 1997, Tala-Heikkilä 2001, Rieder 2003, pp. 337-348). The theory of the transmission of tuberculosis via droplet infection was established in 1897 and confirmed by Wells in the 1950´s (Wells 1955, Roberts and Buikstra 2003, p. 15).

In the 1930´s altogether 88 % of the large population of 56,417 Finnish military recruits were found to be tuberculin positive and in the 1940´s almost 9,000 new cases were detected yearly (Savonen 1937, pp. 67-68, Härö 2000). The battle against tuberculosis rested on diligent health care workers, volunteers and active foundations and associations during the first half of the twentieth century. Intensive health education, work in sanatoria and dispensaries, mass screening by radiography and improving living conditions as well as systematic BCG vaccination of newborns from the 1950´s onwards contributed to the decline of tuberculosis (Härö 2000, Tala-Heikkilä 2003, Teramo 2003). Tuberculosis treatment during the period 1930-1960 included mainly rest, enhanced nutrition and collapse surgery by thoracoplasty, pneumothorax treatment and resection of the cavities. However, no effective cure for tuberculosis or control of the epidemiological situation was available until the discovery of chemotherapy.

After the introduction of streptomycin (SM) in 1945, the basic principles for the most effective treatment were established through various investigations during the next three decades (Mitchison 2005). The present treatment protocol includes a multi-drug combination in which the latest single drug, rifampin (RIF), was introduced as early as in 1965. A new era in tuberculosis control began with the development of molecular diagnostics in the late 1980´s. Moreover, the sequencing of the *Mycobacterium tuberculosis* (*M. tuberculosis*) genome in 1998 has enhanced intensive drug and vaccine investigations (Cole et al. 1998). Until the next major achievements are available the most essential means of tuberculosis control are rapid identification and effective cure of infectious cases (Elzinga et al. 2004).

1.2. Epidemiology

Tuberculosis, along with AIDS and malaria, is one of the leading causes of death among infectious diseases. According to estimates, one third of the world's population is infected with *M. tuberculosis.* Approximately eight million new cases are detected and nearly 2 million people die of tuberculosis each year (Corbett et al. 2003, WHO 2006). Africa has the highest estimated incidence rate (356/100 000), but the majority of TB patients live in the most populous countries of Asia: Bangladesh, India, China, Indonesia and Pakistan (Dye 2006). Tuberculosis incidence is furthermore rising in sub-Saharan Africa due to an HIV-driven epidemic; and multi-drug resistant strains pose a major challenge, especially in areas of the former Soviet Union (Elzinga et al. 2004). In most Western European countries as well as in Sweden, Norway and Denmark over 50 % of new TB patients are foreign born (EuroTB 2006). In 2006 WHO launched the new extensive Stop TB Strategy 2006-2015 for global tuberculosis control, in which one aim by 2015 is to reduce the prevalence of deaths due to TB by 50 % relative to 1990 (Raviglione and Uplekar 2006).

The number of new TB patients and incidences per year in Finland during the period 2000-2005 are presented in Table 1. The incidence of tuberculosis fell below 10/100 000 inhabitants in 2001. In 2005 most patients (73 %) had pulmonary tuberculosis and half of them were infectious. More than half of tuberculosis patients are 65 years or older (National Public Health Institute 2005). While TB has become less common, new cases are mainly detected from various risk groups, of which the most important are elderly people, substance abusers, refugees and close contacts of infectious TB patients (Rajalahti et al. 2004). We have a rather peculiar situation in Finland; while in 2004 the incidence of TB was about 6/100 000, in areas close to our borders as in Russia and in the Baltic countries it was about 40-84/100 000 (Dye 2006, WHO 2006). Moreover, multi-drug resistant (MDR) cases and HIV-TB co-infections are rather common in these countries, whereas in Finland we detect 0-3 MDR cases per year. Hence, increased travel and migration across the borders may create challenges in terms of the TB control in Finland in the future.

Table 1. *Summary of new tuberculosis (TB) cases in Finland 2000-2005 (National Public Health Institute 2005 and 2006a).*

Year	New TB cases (n)	Pulmonary TB cases (n)	Smear positive (%)[1]	Foreign born (%)[2]	MDR-TB[3] cases (n)
2000	537	370	61	8	2
2001	493	316	50	13	4
2002	474	297	46	10	3
2003	413	292	51	12	3
2004	331	230	54	12	0
2005	358	261	50	14	2

[1]The proportion of all new pulmonary TB cases, [2]The proportion of all new TB cases, [3]MDR-TB, multi-drug resistant tuberculosis

1.3. Mycobacterium tuberculosis complex

Tuberculosis is a disease caused by a mycobacterium belonging to the *Mycobacterium tuberculosis* complex. This complex includes several closely related mycobacterium species: *M. tuberculosis, M. bovis, M. africanum, M. microti* and *M. canettii* (van Soolingen et al. 1997, Pfyffer 2003, pp. 67-68). Of these species *M. bovis* causes disease in humans and warm-blooded animals such as cattle and *M. bovis* BCG is used for a vaccine. Different phenotypes of *M. africanum* may be detected in tuberculosis patients in tropical Africa. *M. microti* is mainly a pathogen of small rodents but has also been identified as a pathogen among humans*; M. canettii* has been found to cause lymphadenitis and generalized tuberculosis in humans (van Soolingen et al. 1997, van Soolingen et al. 1998). However, *M. tuberculosis* is the main bacterium inducing disease in humans. It is a slow-growing aerobic organism of 1-5 μm size with a thick cell wall constructed of mycolic acids, which make it acid and alcohol fast. With complicated and sophisticated genetic diversity *M. tuberculosis* has become a master at resisting immune defence and adapting to difficult conditions in various tissues (Cole et al. 1998, Viljanen 2004).

Since the members of *M. tuberculosis* complex are genetically nearly identical, detection of different species with commercial NAA tests is not usually possible. In most countries *M. bovis* infection in humans is rare and has a minor effect in molecular diagnostics; whereas differentiation between tuberculosis and infection caused by BCG vaccination is difficult with NAA tests. Patient history, clinical picture and culture results are key elements in those cases.

1.4. Transmission, infection and disease

Tuberculosis transmission from person to person is primarily airborne. Although smear-negative TB patients have been reported to transmit TB (Behr et al. 1999), in practice a patient is determined to be infectious when acid-fast rods are detected in the smear microscopy of respiratory specimens. Hence, pulmonary and laryngeal tuberculosis are the most infectious forms of the disease. During coughing, sneezing or speaking a person spreads aerosol containing *M. tuberculosis* bacteria, which after the evaporation of water remain in droplet nuclei in the air for prolonged periods of time. Transmission can occur when an exposed individual inhales these droplet nuclei. Those bacteria reaching the alveoli are ingested by local macrophages. If the macrophages are not able to destroy the bacteria, cell mediated immunity reaction is activated and delayed-type hypersensitivity generated, which can be detected by the Mantoux tuberculin test after 3 to 8 weeks (Zellweger 1997, pp. 2-3, Grange 2003, pp. 89-91, Lucas 2003, pp. 76-77).

In addition to local spreading, bacteria are transmitted from the initial pulmonary focus through lymphatics to hilar and mediastinal lymph nodes and by the bloodstream to distant sites. If the spread is uncontrolled, an active disease develops and is called primary tuberculosis (Davies 2003, pp. 107-111). However, if the host's immune response overcomes the bacterial invasion, the bacteria are contained by the macrophages and isolated by caseous granuloma formation and consequently active disease is prevented. This condition is classified as a latent tuberculosis infection (LTBI) (Lucas 2003, pp. 76-78, Ulrichs and Kaufmann 2003, pp. 112-113).

Tuberculosis bacteria are capable of adapting to low oxygen content and use lipids as an energy and metabolic source and subsequently remain dormant in tissues for years and decades (Wayne et al. 1996, Hernández-Pando et al. 2000). Most infected people contain the infection by efficient immune response. However, if the cell-mediated immune system weakens due to various reasons such as HIV, malnutrition, aging and immunosuppressive treatments, an active disease develops. AIDS is the strongest known factor in enhancing activation of TB infection. Postprimary tuberculosis may result from endogenous reactivation or exogenous reinfection and be manifested as pulmonary or extrapulmonary disease (Davies 2003, pp. 111-118, Ulrichs and Kaufmann 2003, pp. 113-124). In the lungs caseous material in granulomas liquefies and may be expelled into the bronchi, resulting in the formation of cavities. In the cavities bacteria multiply effectively in aerobic conditions. Cavities may harbour up to 10^8 bacteria, which can spread to other bronchial segments and be excreted in the sputum, leading to infectivity of the patient (Zellweger 1997, pp. 1-4).

1.5. Pulmonary and extrapulmonary disease

Active tuberculosis disease is designated as pulmonary or extrapulmonary. Pulmonary disease is the most common form of tuberculosis and more than half of these patients transmit the disease; obviously this proportion is dependent on the efficacy of regional TB control policy to identify patients at early stage of the disease. Extrapulmonary tuberculosis is defined as a disease that affects any organ or site outside the pulmonary parenchyma. Its most severe forms are tuberculous meningitis and disseminated tuberculosis, the most common being cervical lymphadenitis and pleural tuberculosis (Ormerod 2003). A patient with extrapulmonary TB is infectious only if aerosol containing plenty of mycobacteria is generated from excretion from the disease site, for example, by treatment measures. The diagnostic procedure for pulmonary TB is mostly straightforward, since chest radiographs and collection of sputum specimens are usually easy to perform, whereas confirmation of extrapulmonary disease is often complicated due to paucibacillary disease and difficulties in obtaining specimens from different sites.

1.6. Symptoms and treatment

The common constitutional symptoms of tuberculosis are high body temperature, weight loss, fatigue and night sweats. Additionally, classical signs of pulmonary tuberculosis include persistent cough, sputum production, haemoptysis, dyspnoea and chest pain. In extrapulmonary disease local signs may be present depending on the organs involved. However, it is not uncommon that TB imitates other diseases and may be neglected in the differential diagnostics in a low-incidence area. On the other hand, patients may only have minor symptoms or be quite symptomless.

Treatment with multiple drugs and for long enough is essential in order to eliminate the bacteria, inhibit the emergence of resistance and prevent relapses. At present the basic chemotherapy for drug-sensitive tuberculosis is accomplished with three to four drugs (isoniazid (INH), rifampin, pyrazinamid (PZA) and ethambutol (EMB)) for the first two months and continued with INH and RIF for four months (ERS Task Force 1999). Infectious patients are normally isolated for two weeks in hospital. The microbiological cure is confirmed by sputum cultures during and at the conclusion of the treatment provided that patients can indeed expectorate sputum samples.

2. Nontuberculous mycobacteria

In addition to the *M. tuberculosis* complex, the mycobacterium genus consists of nearly 100 species identified to date named nontuberculous mycobacteria (NTM). These bacteria are found in abundance in air, water, food and soil, but only few species may cause disease and mainly in immunocompromised humans. The most common pathogens are *Mycobacterium avium, M. intracellulare, M. kansasii*, *M. malmoense* and *M. xenopi* (Viljanen et al. 2005). One species, *M. branderi*, named by Elias Brander, was identified by a Finnish group and has been shown to be a potential pathogen for humans (Brander et al. 1992, Koukila-Kähkölä et al. 1995). NTM diseases are detected in lungs, lymph nodes, skin and soft tissues. Disseminated disease is common especially in patients with cell-mediated immunodeficiency like an HIV infection. Pulmonary disease resembles tuberculosis but is not transmitted between humans (Katila et al. 2004, Viljanen et al. 2005). Thus no isolation of patients and contact investigations are needed. NTM may, however, interfere with TB diagnostics, since these bacteria are detected by the smear test, which does not differentiate *M. tuberculosis* from NTM as discussed in the next section. At present NTM isolations are more prevalent in Finland than *M. tuberculosis* complex. The incidence of isolated NTM species was 9/100 000 in 2005 (National Public Health Institute, National Register of Infectious Diseases 2005).

3. Diagnostics of tuberculosis

3.1. Clinical picture and radiology

The diagnosis of tuberculosis is dependent on the physician's awareness and clinical experience in predicting the disease and interpreting results of the investigations made. It is based on patient history (previous exposure and risk factors for TB), symptoms, physical examination and radiological findings consistent with TB, and in some cases histological signs consistent with tuberculosis. The Mantoux test may confirm the infection; however, test results are difficult to interpret in a population vaccinated with BCG. The most important tests to detect pulmonary tuberculosis are microscopic examination and culture of the sputum specimens. Positive smear result reveals infectiousness and microbiological confirmation is obtained by the isolation of *M. tuberculosis* bacteria in the culture. In some individual cases bacteriological tests remain negative, and the diagnosis is confirmed by positive response to TB treatment.

Chest radiography is the first choice in the assessment of patients with suspected pulmonary TB and may provide adequate information for the diagnosis. Characteristic findings of active postprimary TB are cavitary or fibronodular lesions in the apical and posterior segments of the upper lobe or in the superior segment of the lower lobe. Further, pleural effusion may be involved and tuberculomas may appear as single round densities (Woodring et al. 1986, Lee 1996). A normal chest radiograph does not, however, exclude pulmonary or miliary TB (Woodring et al. 1986, Vasankari 1998). Findings identified in inactive disease are residual fibrotic scarring, cavities, loss of lung volume, pleural thickening and calcified mediastinal lymph nodes, which remain unchanged at follow-up. Distinguishing reliably between other diseases and active or inactive tuberculosis is difficult (Woodring et al. 1986). High resolution computed tomography (HRCT) is of value in specifying the findings, detecting minimal changes and making a distinction between active and inactive TB disease. Centrilobular nodules and/or branching linear structures, macronodules, cavities and consolidation as well as tree-in-bud appearance are found in active disease, whereas fibrotic changes, traction bronchiectasis and bronchovascular distortion are typical of inactive disease (Hatipoğlu et al. 1996, Lee et al. 1996).

3.2. Specimens

A diagnosis of pulmonary tuberculosis can be confirmed from respiratory specimens such as sputum, bronchial washes and bronchoalveolar lavage. Sputum samples are the most important and easiest to obtain. If sputum cannot be expectorated, it can be induced in clinics where transmission risks are minimized (Li et al. 1999). Sputum induction has been shown to have a diagnostic yield comparable to fiberoptic bronchoscopy (Conde et al. 2000) and to be safe; the only potential adverse effect is bronchospasm in patients with asthma or chronic obstructive pulmonary disease (Conde et al. 2000, Menzies 2003). In extrapulmonary cases various specimens such as fluid, pus, biopsies, urine, bone marrow and other tissue samples from different sites are obtained depending on the nature of the tuberculosis.

3.3. Smear microscopy

Microscopic examination of acid-fast bacteria (AFB) is also called the smear test. As with all diagnostic TB tests, multiple specimen collection, preferably three specimens, is important due to irregular shedding of bacteria into the sputum. Briefly, bacteria are stained with either carbolfuchsin (Ziehl-Neelsen and Kinyoun methods) or fluorochrome (auramin-rhodamin and acridine orange methods) dyes and decolorised by acid-alcohol procedure. The remaining acid-fast bacteria are detected by light microscopy after carbolfuchsin staining and by fluorescence microscopy when using the fluorochrome technique (Pfyffer 2003, pp. 70-71, Eskola and Soini

2004). Compared to conventional microscopy, the advantages of fluorescence microscopy are shorter examination time due to lower magnification and increased sensitivity in paucibacillary specimens, whereas the disadvantages are higher implementation and maintenance costs (Bennedsen and Larsen 1966, Ba 1999, Woods 2002). Good laboratory practice includes screening of specimens by fluorescence microscopy, confirmation of positive smear and culture results by Ziehl-Neelsen method as well as reporting the smear results within 24 hours (Hale et al. 2001).

The smear test is a rapid, simple and inexpensive test to assess TB diagnosis, and currently the only test to confirm the infectivity of the patient. However, the detection limit in sputum is estimated to be 10^4 bacilli per ml and the overall sensitivity has ranged from 22 % to 78 % (Daniel 1990, Gordin and Slutkin 1990). Moreover, no distinction between *M. tuberculosis* and nontuberculous mycobacteria is attainable by smear; and organisms such as *Nocardia, Cryptosporidium* and *Legionella micdadei* may be detected in acid-fast smear impairing the specificity of the test (Pfyffer 2003, p.71, Viljanen et al. 2005, pp. 142-143).

3.4. Culture

Due to the slow-growing nature of mycobacteria contamination of the culture by other bacteria is inevitable without decontamination of the specimen prior to cultivation. N-acetyl-L-cysteine (NALC) is used to liquefy the specimen and sodium hydroxide (NaOH) to destroy other competing bacteria. However, Yajko and colleagues (1995) found that after NaOH pre-treatment only 11 to 20 % of *M. tuberculosis* survived compared to nondecontaminated samples. Therefore a careful protocol is essential not to destroy mycobacteria during decontamination (Eskola and Soini 2004). At present the standard culture procedure consists of a combination of a solid and liquid media because neither medium recovers all isolates (Cruciani et al. 2004). Solid media such as egg-based Löwenstein-Jensen or agar-based Middlebrook 7H11 require 3 to 8 weeks for sufficient growth of mycobacteria and slants are checked weekly (Woods 2002).

The precursor of current liquid media culture is the semi-automated BACTEC 460TB System (Becton Dickinson), in which the radio-labeled palmitic acid is metabolized to $^{14}CO_2$ by the growing bacteria and is monitored by the instrument. However, problems with radioisotopes have led to the development of safer non-radiometric liquid media. Fully automated culture systems with continuous monitoring such as BACTEC Mycobacteria Growth Indicator Tube (MGIT) 960 (Becton Dickinson) and MB/BacT Alert 3D (Organon Teknika) utilize a colorimetric CO_2 sensor to detect the growth of mycobacteria. These most advanced culture methods are competitive with the BACTEC 460TB System, save labor and are more sensitive than conventional solid media (Pfyffer et al. 1997, Pfyffer 2003, pp. 73-75, Cruciani et al. 2004). Additionally, the detection time is fairly short, ranging from 10 to 14 days (Hanna

et al. 1999, Kanchana et al. 2000, Katila et al. 2000). Subsequently, the BACTEC 460TB System has generally been replaced by the advanced methods.

Currently, culture is the gold standard in the diagnosis of tuberculosis. Moreover, it provides mycobacteria for further species identification and antimicrobial susceptibility testing, and it is the best available means to assess microbiological response during chemotherapy (Pfyffer 2003, p. 73).

3.5. Species identification

Biochemical techniques such as chromatographic methods have proved slow, labour-intensive and insensitive and have mostly been replaced by molecular biology methods (Figure 1). Species identification is based on species-specific labelled deoxyribonucleic acid (DNA) probes, which hybridize with the ribosomal ribonucleic acid (rRNA) released from the mycobacteria. Results are detected with a luminometer and obtained in two hours. Commercial probes (AccuProbe; Gen-Probe Inc) are available for *M. tuberculosis* complex and NTM such as *M. avium, M. intracellulare, M. gordonae* and M. *kansasii*. Each species is tested for separately one by one.

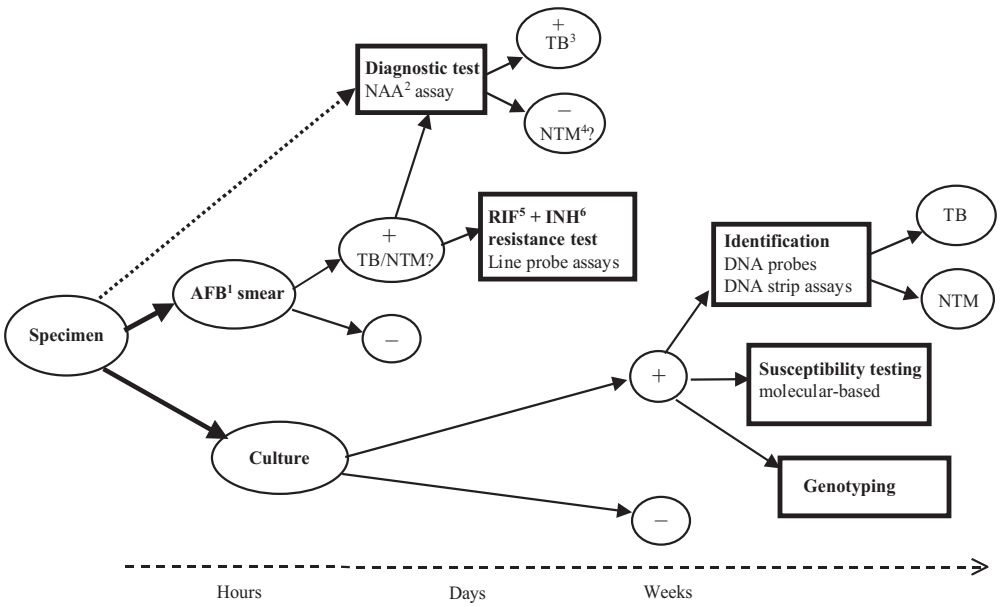

Figure 1. *Molecular methods (shown in boxes) in the diagnostics of tuberculosis.*

[1]AFB, acid-fast bacteria; [2]NAA, nucleic acid amplification; [3]TB, tuberculosis; [4]NTM, nontuberculous mycobacteria; [5]RIF, rifampin; [6]INH, isoniazid

Advanced commercial versions of serial DNA probes (GenoType, Hain Lifescience and InnoLiPA, Innogenetics) have recently been introduced. DNA strip tests allow simultaneous detection of up to 13 to 16 mycobacterial species in approximately 5 hours (Tortoli et al. 2003, Eskola and Soini 2004). The method is based on the reverse hybridization of PCR amplicons to their complementary probes and subsequently simultaneous detection and identification of mycobacteria. Sarkola and colleagues (2004) found an overall agreement of 89 % between GenoType and the two reference methods (AccuProbe and 16S rDNA sequencing) in the routine identification of mycobacteria. The GenoType assay test was shown to be more sensitive than the AccuProbe test in 12 samples, which was estimated to derive from culture growth insufficient to be identified by AccuProbe (Sarkola et al. 2004).

The current gold standard for mycobacterial identification is the sequencing of the 16S RNA gene. In this gene most mycobacterial species have sequence diversity, which allows definitive identification of known species and also detection of new mycobacterial species. This procedure is time-consuming and demands resources and experienced personnel, and is therefore mainly sustained in reference laboratories and for research purposes (Soini and Musser 2001).

3.6. Histology

Although bacteriology is the key examination for confirming the diagnosis of TB, histology is an important aid, particularly in the diagnosis of various extrapulmonary forms. Tissue samples for microscopic examination may be obtained by biopsy, surgical interventions or in the worst case in autopsy. The specific lesion for tuberculosis in subacute phase is the epithelioid giant cell follicle with caseating necrosis. Combining bacteriological techniques to histology, such as staining and culture of tissue samples, can enhance the confirmation of extrapulmonary tuberculosis.

4. Molecular diagnostic tests in tuberculosis

4.1. PCR method

Polymerase chain reaction was invented in the 1980`s (Saiki et al. 1985, Mullis et al. 1987, Saiki et al. 1988). The inventor, Kary Mullis, was awarded the Nobel Prize in chemistry in 1993. Saiki and co-workers (1985) first described the amplification technique applied to

ß-globine sequences. PCR is a method by which a nucleic acid sequence is exponentially amplified by polymerase-catalyzed chain reaction in vitro (Mullis et al. 1987). It is based on species specific sequences. Identification of specific repetitive DNA sequences is mainly used since multiple copies theoretically enhance the sensitivity of the test. By knowing the ends of these sequences complimentary oligonucleotides, referred as primers, can be synthesized. Duplex DNA is denaturated and the primers anneal to the ends of opposite single strands to start extension and production of complementary DNA from synthetic deoxynucleotides (dNTPs). Amplification is catalyzed by DNA polymerase enzyme, which works most efficiently in high temperatures. With the introduction of the heat stable DNA polymerase derived from *Thermus aquaticus* (*Taq* polymerase) the procedure was simplified, and automated thermocycling was obtainable (Saiki et al. 1988). Further, characterization of amplified species was enhanced with the labelled oligonucleotide probes (Hance et al. 1989).

During the first half of the 1990`s the development of amplification techniques took place mainly in molecular biology laboratories. Studies were performed on various targets of *M. tuberculosis* complex including genes encoding mycobacterial proteins such as 65-kDa antigen (Brisson-Noel et al. 1989, Brisson-Noel et al. 1991), 32-kDa antigen (Soini et al. 1992) and MPB64 (Shankar et al. 1990) and repetitive sequences such as IS6110 (Thierry et al. 1990a, Thierry et al. 1990b). Other amplification techniques and commercial nucleic acid amplification (NAA) tests were developed, and gradually NAA assays were implemented in clinical microbiology laboratories. At present in-house NAA tests are nevertheless widely used due to the high costs of the commercial assays.

4.2. Commercial NAA assays

The Cobas Amplicor (Roche) system is a single unit combining five instruments (automated pipettor, incubator, thermal cycler, wash station and photometer), which enable automated amplification and detection of the *M. tuberculosis* complex. This qualitative test amplifies target DNA, which is a 584-bp segment of the 16S ribosomal RNA gene (DiDomenico et al. 1996). The test includes four steps: specimen preparation, PCR amplification, hybridization and detection. In brief, specimens are liquefied and decontaminated with NALC-NaOH. A portion of 50 µl of the processed specimen is added to the amplification mixture in amplification tubes containing *Taq* polymerase, biotinylated primers and abundant dNTPs including deoxyadenosine, deoxyguanosine, deoxycytidine and deoxyuridine (dUTP) in place of deoxythymidine (DiDomenico et al. 1996). The amplification process includes denaturation of the double stranded DNA, annealing of the primers and extension of the amplicon sequence, which occur at different temperatures. The procedure is repeated for the required number of cycles, and consequently the copies of the original DNA sequence increase exponentially. Further, after hybridization of *M. tuberculosis*-specific DNA probe, the detection is accomplished

by a colorimetric reaction measured with a photometer at wavelength of 660 nm. Absorbance values ≥ 0.35 are scored positive (DiDomenico et al. 1996, Piersimoni and Scarparo 2003).

False positive results due to carryover contamination from previous amplicons are prevented by AmpErase reagent containing the enzyme uracil-N-glycosylase, which destroys dUTP containing amplicons (Soini and Viljanen 1997). AmpErase is inactivated at cycling temperatures leaving newly formed amplicons unaffected. Moreover, false negative results are addressed by an internal control, which is included in each run to detect inhibitory substances. The result is obtained in 6.5 hours after specimen preparation. The commercial test system simplifies laboratory setup and decreases hands-on work time during the procedure. On the other hand implementing and performing the test system requires financial investments and extensive laboratory resources.

Transcription-mediated amplification (TMA) is an isothermal system amplifying rRNA (16S rRNA) by DNA intermediates. Briefly, the promoter-primer binds to the target rRNA and the reverse transcriptase enzyme creates DNA copy of the target. rRNA is degraded from the RNA-DNA duplex and the primer 2 anneals to the DNA and new DNA is made. Subsequently DNA-directed RNA polymerase transcribes RNA amplicons from the DNA template. New synthetised amplicons re-enter the TMA process, and repeated replication cycles produce a billion-fold amount of RNA amplicons. The amplicon products are detected with an acridinium ester-labelled DNA probe in a hybridization assay and the results are read by the luminometer (Soini and Viljanen 1997). The commercial TMA assay (AMTD2, GenProbe) differs from the Cobas Amplicor test in some aspects (Table 2). Firstly, thousands of copies of the target rRNA are present in mycobacterial cells compared to 10 to 20 copies of target DNA used in the PCR assay.

Table 2. *Characteristics of the Cobas Amplicor and Amplified Mycobacterium Tuberculosis Direct (AMTD2) assays.*

Feature	Cobas Amplicor	AMTD2
Amplification method	PCR[1]	TMA[2]
Target	16S r[3]DNA	rRNA
Sample volume (microliters)	50	450
Prevention of carryover contamination	Yes	No
Internal control for inhibitors	Yes	No
Assay time after specimen decontamination (hours)	6.5	3.5
Number of samples per run	96	50

[1] PCR, polymerase chain reaction; [2] TMA, transcription-mediated amplification; [3] ribosomal.

Secondly, 450 µl instead of 50 µl of prepared specimen is used in the assay (Bodmer et al. 1996). Theoretically these might increase the sensitivity of the TMA test in clinical specimens. However, in contrast to the PCR assay, internal control for inhibitory substances is not included in the TMA assay.

Ligase chain reaction (LCx) is a method based on DNA amplification. Two primers attach to each strand leaving a gap in between, which is filled by the action of DNA polymerase and the primers are linked together by ligase. The first pair of oligonucleotides acts as a template for new complementary oligonucleotides. Detection of the amplicons is carried out by microparticle enzyme assay with the LCx fluorimetric analyzer. The LCx MTB assay (ABBOTT LCx probe system) has gone through various modifications. This test has no internal control and the main shortcomings have been with inhibitory susbstances and lack of sensitivity. The test was withdrawn from the European market in 2002.

The recently developed commercial assay is the ProbeTec ET DTB (Becton Dickinson) test based on the strand displacement amplification (SDA). It is an automated isothermal method characterized by simultaneous DNA amplification and real-time fluorometric detection of the amplicons. An internal control to detect the presence of inhibiting substances is included in each run. The test performance time is approximately 4 hours after specimen preparation (Piersimoni and Scarparo 2003). Another advanced qualitative NAA test is the Geno Type Mycobacteria Direct (Hain Lifescience) test. This method includes RNA isolation, isothermal amplification and detection by reverse hybridization. It is based on a DNA strip technology, and in addition to *M. tuberculosis* complex it permits simultaneous detection of *M. avium, M. intracellulare, M. kansasii* and *M. malmoense.*

4.3. Performance of the NAA tests

For two decades NAA techniques have been continuously refined and improved. The analytical sensitivity of the Amplicor PCR test was determined to be approximately 10 organisms/100 µl by Jackson and co-workers (1996). The sensitivity of the AMTD test was estimated even better since ribosomal RNA is present with thousands of copies in cells. Yajko and colleagues (1995) showed that the minimum number of *M. tuberculosis* colony forming units (CFU) detected from samples by the Amplicor PCR was 42 CFU in nondecontaminated samples, corresponding to 8 CFU in decontaminated samples. Moreover, although decontamination with NALC-NaOH treatment was found to kill approximately 80 % of the mycobacteria, it did not affect the sensitivity of the PCR test, indicating that PCR may detect nonviable mycobacteria from sputum. Performance of NAA tests has been assessed in numerous studies of which some are presented in Table 3.

Table 3. *Summary of selection of studies evaluating nucleic acid amplification (NAA) tests for detection of Mycobacterium tuberculosis complex from clinical specimens.*

Study	NAA method	Patients	Specimens/ type	TB[2] culture +/ smear +	NTM[3] culture +/ smear +	Sensitivity	Specificity	PPV[4]	NPV[5]	Sensitivity for	
										Smear + specimens	Smear - specimens
		(n)	(n/ R, E)[1]	(n/n)	(n/n)	(%)	(%)	(%)	(%)	(%)	(%)
Reischl et al. 1998	Cobas Amplicor	807	643/R	57/44	35/17	84	99	NA[6]	NA	95	50
			506/E	39/25		82	98	NA	NA	100	61
Levidiotou et al. 2003	Cobas Amplicor	3321	7722/R	254/195	15/NA	84	100	94	99	97	49
			1451/E	18/0	1/NA	50	100	NA	NA	NA	50
Michos et al. 2006	Amplicor PCR	2296	2296/R+E	113/36	25/14	80	98	78	99	97	75
Gamboa et al. 1998	AMTD2[7]	515	410/R	95/48	26/19	95	100	100	98	100	83
			272/E	68/21	9/3	87	100	100	98	100	89
Chedore and Jamieson 1999	AMTD2[7]	NA	616/R + 207/E	245/230	247/226	100	100	97	100	100	100
Coll et al. 2003	AMTD2[7]	3360	3308/R	260/185	163/48	91	100	99	99	99	70
			1350/E	73/26	12/NA	67	100	98	98	94	52
Viinanen et al. 2000	LCx[7]	86	247/R	31/24	10/9	84	98	84	98	96	43
Maugein et al. 2002	ProbeTec	478	547/R	69/43	10/6	89	98	NA	NA	100	76
			74/E	8/2	2/NA	89	97	NA	NA	100	86
Rusch-Gerdes and Richter 2004	ProbeTec	731	735 R + 396 E	125/39	18/2	90	97	78	99	100	86

[1]R, respiratory specimens; E, extrapulmonary specimens; [2]TB, tuberculosis; [3]NTM, nontuberculous mycobacteria; [4]PPV, positive predictive value; [5] NPV, negative predictive value; [6] NA, not available; [7] Not including internal control.

The performance of NAA tests is excellent in smear-positive respiratory specimens and varies only slightly depending on study populations, whereas for smear-negative paucibacillary specimens the sensitivity is considerably lower (Table 1). The combination of culture and clinical correlation is used as the reference standard since specimen quality, preparation procedures and contamination problems may affect the recovery of mycobacteria in culture tests. Despite the capability of NAA tests to detect noncultivable organisms, the overall sensitivity of NAA assays has been found to be lower compared to culture (Woods 2002, Piersimoni 2003). Commercial NAA assays have been studied in nonrespiratory specimens as well (D´Amato et al. 1996, Shah et al. 1998), although they are primarily intended to be used with respiratory specimens. The results have varied considerably, at least partly due to nonstandardized specimen processing and uneven proportions of different types of specimens between studies (Woods 2002).

Comparison between different NAA assays is difficult due to variable study populations and specimen combinations as well as disparate specimen preparation and amplification methods. Further, a greater proportion of smear-positive samples in a study material enhances the sensitivity of the NAA test. Noordhoek and colleagues (1994) found a wide variation in sensitivity and specificity when seven laboratories were evaluated in using an IS6110 based in-house NAA assay for a set of 200 specimens. Problems such as cross-contamination and inhibition of amplification as well as unfamiliarity with the amplification method used were detected. Subsequently, an interlaboratory study involving 30 laboratories in 18 countries was conducted (Noordhoek et al. 1996). Each laboratory (8 laboratories used commercial tests) tested a set of 20 specimens with their own protocol of amplification. However, only 5 laboratories achieved the correct results, and reliability was not associated with any specific NAA method. In the recent quality control study with 82 participating laboratories from 23 countries (62 % used commercial tests) the performance values of the NAA-tests had improved substantially, but the results were found to be more user-dependent than method-specific (Noordhoek et al. 2004). The authors have thus underlined the need for standardized procedures and quality control measures throughout the entire specimen preparation and testing process.

Appropriate comparison of different NAA methods is obtainable when conditions in specimen processing are equal. Studies comparing different NAA methods in the same study material are presented in Table 4. The overall agreement with the commercial NAA assays was high, and generally no statistically significant differences were observed between the methods. However, there were differences between NAA tests relating to the presence of inhibitory samples; the need for internal control in each test was stressed (Scarparo et al. 2000, Piersimoni et al. 2002).

Table 4. *Summary of studies comparing different nucleic acid amplification (NAA) assays for detecting Mycobacterium tuberculosis complex from specimens of the same patient population.*

Study	NAA method	Patients	Specimens	Culture +/ Smear + specimens	Sensitivity	Specificity	PPV[2]	NPV[3]	Sensitivity for	
									Smear + specimens	Smear - specimens
		(n)	(n/R, E)[1]	(n/n)	(%)	(%)	(%)	(%)	(%)	(%)
Vuorinen et al. 1995	Amplicor PCR[4]	243	256/R	26/21	83	100	100	98	NA[5]	NA
	AMTD[4]				86	100	100	98	NA	NA
Della-Latta and Whittier 1998	Amplicor PCR[4]	156	1385/R	62/NA	97	100	NA	NA	97	96
	AMTD[4]				97	100	NA	NA	100	93
Dalovisio et al. 1996	Amplicor PCR[4]	259	428/R	91/49	80	96	86	94	NA	70
	AMTD[4]				84	98	92	95	NA	77
	IS6110-PCR[4]				83	99	98	95	NA	77
Wang and Tay 1999	Cobas Amplicor	230	230/R	72/66	96	100	100	98	97	100
	AMTD2[4]				99	99	99	99	100	100
	LCx[4]				100	99	99	100	100	100
Scarparo et al. 2000	AMTD2[4]	323	296/R	114/97	86	100	100	90	92	66
			190/E	33/25	83	100	100	96	88	75
	Cobas Amplicor		296/R	114/97	94	100	100	97	99	75
			190/E	33/25	85	100	100	96	96	69
Piersimoni et al. 2002	AMTD2[4]	253	331/R	91/76	88	99	NA	NA	NA	NA
		149	184/E	30/22	74	100	NA	NA	NA	NA
	ProbeTec		331/R	91/76	95	100	NA	NA	NA	NA
			184/E	30/22	92	100	NA	NA	NA	NA

[1]R, respiratory; E, extrapulmonary; [2]PPV, positive predictive value; [3]NPV, negative predictive value; [4]Not including internal control; [5]NA, not available.

Table 5. *Summary of studies evaluating nucleic acid amplification (NAA) assays for detecting tuberculosis (TB) patients.*

Study	NAA method	Patients	Specimens/ type	Culture +/ Smear + TB cases	Se[2]	Sp[3]	PPV[4]	NPV[5]	Sensitivity for	
									Smear + patients	Smear - patients
		(n)	(n/ R, E)[1]	(n/n)	(%)	(%)	(%)	(%)	(%)	(%)
Bennedsen et al. 1996	Amplicor PCR	3738	7194/R	293/204	88	99	92	99	99	64
Cohen et al. 1998	Amplicor PCR	85	316/R	27/12	74	93	NA[6]	NA	100	53
Bergmann et al. 1999	AMTD2	486	995/R	22/10	91	99	83	100	100	83
Al Zahrani et al. 2000	Amplicor PCR	487	NA/R	44/10	42	100	NA	NA	NA	NA
Catanzaro et al. 2000	AMTD2	338	NA/R	65/43	83	97	88	95	NA	NA
Cheng et al. 2004	IS6110 PCR	155	224/R+E	112/35	81	100	NA	NA	NA	NA

[1] R, respiratory; E, extrapulmonary; [2] Se, sensitivity; [3] Sp, specificity; [4] PPV, positive predictive value;
[5] NPV,negative predictive value; [6] NA, not available.

It has been widely recognized that in many studies more than one specimen per patient is included in calculations of performance values. On the other hand, some studies have reported that multiple specimen collection enhances the sensitivity of both conventional as well as the NAA tests. Thus patient based evaluations have been performed along with the laboratory studies. Again study protocols and populations differ considerably from each other as shown in Table 5.

The sensitivity of the NAA assay with smear-negative patients was shown to be suboptimal in the study by Cohen and co-workers (1998). They found that false negative NAA results were detected in specimens with fewer than 20 colonies growing in the culture. Similarly, the difficulty of diagnosing minimal pulmonary tuberculosis was shown in the study by Al Zahrani and colleagues (2000). Their study population included patients with negative smears or patients referred to sputum induction because spontaneous sputum was not obtainable. The sensitivity of the NAA test was low, as expected in a paucibacillary disease, and clinical judgment played an important role in the diagnosis of TB. However, no false positive NAA results were found and consequently the specificity was high. In contrast to these studies, a good sensitivity for the NAA test in all patients as well as in smear-negative patients was found in the study by Bergmann and co-workers (1999). This is at least partly explained by the highly selected study population including only prison inmates.

4.4. NAA testing in selected populations and circumstances

NAA tests have been studied in various populations, conditions and in selected specimen groups. Conventional tests have limitations in diagnosing tuberculous meningitis or pleuritis, and therefore the diagnostic accuracy of NAA assays in these diseases has been of interest. According to a systematic review and meta-analysis of 40 studies, the overall sensitivity of commercial NAA tests (14 studies) for TB pleuritis was 62 % (95 % confidence interval 43-77 %) and the specificity 98 % (95 % CI 96-98 %) (Pai et al. 2004). For TB meningitis the sensitivity and specificity for commercial NAA tests (14 studies) in the meta-analysis were 56 % (95 % CI 46-66 %) and 98 % (95 % CI 97-99 %) respectively (Pai et al. 2003). Estimates for in-house NAA tests could not be determined in either meta-analysis due to significantly heterogeneous test results. Both systematic reviews suggest that commercial NAA assays have a potential role in confirming pleural or meningeal TB disease. However, because of their overall low sensitivity they cannot exclude the disease with certainty (Pai et al. 2003, Pai et al. 2004). Salian and colleagues (1998) showed that NAA testing could increase diagnostic accuracy in histological specimens. They reported a sensitivity of 74 % and specificity of 100 % for in-house PCR in a study of 60 tissue specimens.

Smith and co-workers (1997) obtained good NAA results in a smear-negative prison population. The Se, Sp, PPV and NPV for smear-negative specimens were 88 %, 100 %, 96 % and 99 % respectively. However, a substantial portion of the study specimens was collected from patients receiving antituberculous chemotherapy. In the study by Laifer et al. (2004) 103 specimens from 29 suspects among 3,119 war refugees were tested by smear, culture and PCR after initial radiographic screening. All culture-positive TB patients had at least one positive PCR result, and hence three respiratory specimens per patient were recommended for PCR testing.

In a population of HIV infected subjects an in-house PCR was capable to detect *M. tuberculosis* DNA in urine specimens from all 13 HIV patients with microbiologically confirmed active pulmonary tuberculosis (Aceti et al. 1999). Only two of the urine specimens were positive by culture, suggesting a low bacterial load and/or nonviable organisms in the samples tested. Another study with HIV patients was performed in Kenya (Kivihya-Ndugga et al. 2004). Altogether 35 % of those attending HIV tests were HIV-positive, and the prevalence of TB in the study population was 57 %. The sensitivity of the Amplicor PCR was 93 %, but specificity only 84 %. Since no cross-contamination was detected, it was explained by that solely culture was used as a gold standard. Clinical follow-up data was not available for the PCR-positive culture-negative patients. The authors also concluded that the main problems with PCR testing in a developing country were maintenance of the equipment and provision of continuous supplies as well as costs of the PCR assay.

The difficulties of developing countries in performing NAA testing were revealed in another study as well. An interlaboratory study with in-house PCR was performed in six Latin American countries (Suffys et al. 2000). The laboratories received 30 blind samples with different concentrations of tuberculosis bacteria. Both sensitivity and specificity of PCR were found unsatisfactory and varied considerably. None of the laboratories performed detection of inhibition, and neither negative nor positive controls were used. Further, cross-contamination leading to false positive results was detected. The laboratories were shown to have problems in maintaining high level of facilities and expertise in performing NAA tests and producing reliable results.

4.5. Limitations of the NAA tests

The main concerns in evaluating the performance of NAA assays are false negative and false positive results (Table 6). Regardless of the NAA test, one of the most significant factors affecting the sensitivity of the test is a low number of bacteria in samples tested. Further, mycobacteria may form clumps and not be uniformly distributed in the specimens. Those relating to the assay protocol are loss of bacteria during sample preparation and small sample volume (50-500 µl) used for the test. Specimens may contain different inhibitory substances such as blood, heparin or detergents. Although inhibition is detected in both pulmonary and extrapulmonary samples, ranging from less than 1 % up to 20 %, the phenomenon is more common in the latter (Eing et al. 1998, Bogard et al. 2001, Rimek et al. 2002, Honoré-Bouakline et al. 2003). Extensive washing of specimens and multiple step extraction methods to purify DNA have been shown to decrease inhibition rates (Woods 2002, Honoré-Bouakline et al. 2003). However, no method has resolved the problem completely and an internal control in each amplification run has proved essential. In case of inhibition the NAA test is repeated after dilution of the specimen. If inhibitory substances are not removed, testing of another sample from the same patient is recommended.

Table 6. *Factors leading to false results by the nucleic acid amplification (NAA) tests.*

False positive results	False negative results
Cross-contamination	Small number of bacteria in a sample
Dead DNA material from past tuberculosis	Clumping of bacteria in a specimen
Too low cutoff values for positive results (AMTD[1])	Inhibition
Mycobacterium leprae (AMTD)	Small sample volume

[1] AMTD, Amplified Mycobacterium Tuberculosis Direct test.

Genotyping of *M. tuberculosis* strains has enabled the detection of cross-contamination in the laboratory (Della-Latta and Whittier 1998). In the study by Jasmer and colleagues (2002) 2 % of the cultures were false positive accounting for 22 % of the patients with a single positive culture. Cross-contamination in NAA testing may result from genomic DNA or RNA from other specimens or cultures during initial specimen processing or sample preparation, or from amplification products generated in the laboratory (Roth et al. 1997, Cohen et al. 1998). It can also happen outside the laboratory via contaminated clinical equipment such as the bronchoscope, but may be avoided by adequate washing of the bronchoscope (Kaul et al. 1996, Shim et al. 2002). High expertise and skilled personnel as well as strict contamination control measures in laboratories are needed to prevent cross-contamination (Roth et al. 1997, Della-Latta and Whittier 1998).

False positive results have been detected in extrapulmonary specimens from immunocompromised patients who do not have active tuberculosis. The persistence of dormant *M. tuberculosis* in sites such as lymph nodes, lung and bone marrow has been suggested as an explanation (Honoré-Bouakline et al. 2003). This is supported by Hernández-Pando and coworkers (2000), who demonstrated that DNA of *M. tuberculosis* was detected in the lung tissues of patients who had neither evidence of active tuberculosis before death nor any histopathological signs of tuberculosis in lungs at necropsy. Most (70 %) of these PCR-positive patients had died of diseases causing immunodeficiency. Further, modifying the detection cutoff of the test for the positive result by increasing the upper limit of the equivocal zone decreased the rate of false positive results and enhanced specificity (Coll et al. 2003). Finally, Chedore and colleagues (2006) observed false positive results in the AMTD test caused by *Mycobacterium leprae* both in a clinical sample and subsequently in processed culture material from mice containing *M. leprae* $\geq 10^5$ bacteria per ml.

4.6. The role of NAA testing in diagnosing TB

The unquestioned value of NAA testing is the rapidity of the test compared to culture. Further, the sensitivity of the test is very good in smear-positive specimens and since NAA test results remain negative in samples containing NTM, NAA testing is useful in differentiating *M. tuberculosis* from other mycobacteria (Smith et al. 1997, Chedore and Jamieson 1999, Scarparo et al. 2000, Bogard et al. 2001). In smear-negative specimens the sensitivity of NAA assays is variable and more modest, and NAA assays are not recommended for routine use with smear-negative patients. The greatest diagnostic accuracy of smear-negative pulmonary TB has been found in studies including bronchial specimens (Sarmiento et al. 2003). The clear advantages of commercial NAA tests over in-house tests are standardized reagents, highly automated processing steps and longer hands-off time of the personnel (Reischl et al. 1998). Despite optimistic expectations, NAA testing has not replaced conventional smear and culture tests, since smear is moreover needed for the assessment of infectiousness and culture for strain identification, susceptibility testing and genotyping of different strains (Figure 1).

In 1999 the U.S. Food and Drug Administration (FDA) approved the AMTD for testing smear-negative respiratory specimens. The AMTD and Cobas Amplicor assays had been earlier approved for testing smear-positive respiratory specimens. According to the guidelines of the Centers for Disease Control and Prevention (CDC, 2000), three sputum specimens on different days are recommended to be collected for AFB smear and culture; the use and summary of basic interpretation of NAA testing is shown in Figure 2. It is emphasized that NAA testing does not replace conventional tests and clinical judgement. Criticism has been expressed that since quality of sputum is variable, the requirement for two positive NAA results for the diagnosis of smear-negative tuberculosis may delay diagnosis (Woods 2002). Moreover, combining the results of an experienced laboratory and appropriate clinical judgment of practitioners would be adequate to base TB diagnosis on a single positive NAA result.

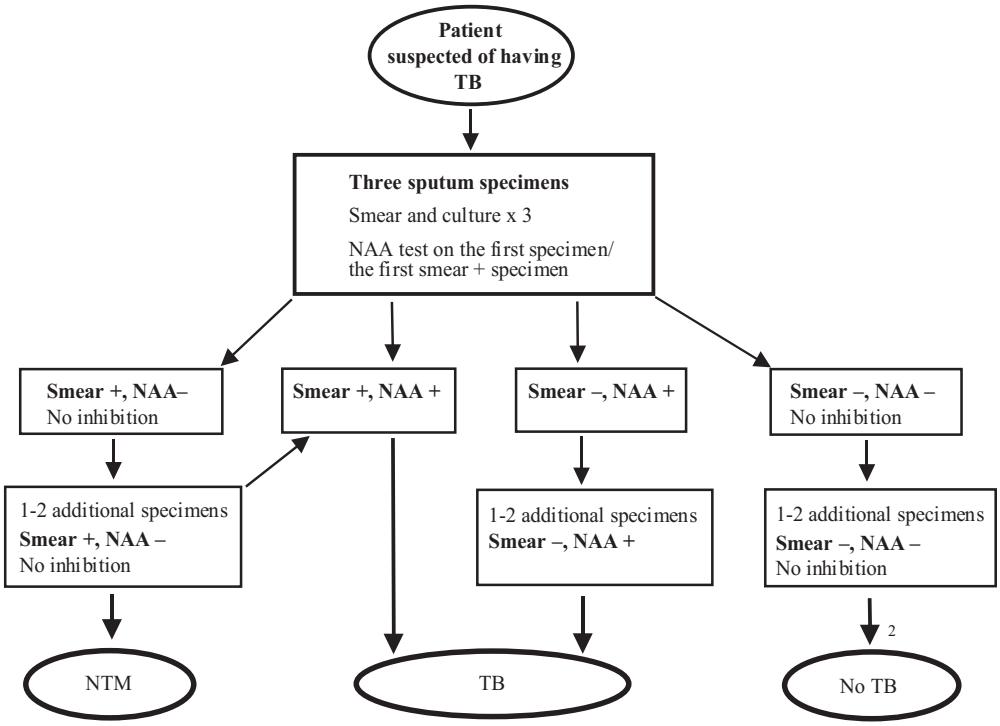

Figure 2. *The use and interpretation of nucleic acid amplification (NAA) tests in patients suspected of having tuberculosis (TB) based on the Centers for Disease Control and Prevention (CDC) recommendation.*

[1]NTM, nontuberculous mycobacteria; [2]Does not definitely exclude active pulmonary TB.

5. Economic evaluation

5.1. General aspects

Resources in health care are limited and thus not all desired activities and methods can be applied in practice. Therefore, choices must be made and economic analysis is one important means to assess the feasibility of a new method for clinical use. Economic evaluation may be broadly defined as the systematic assessment of costs and consequences. It deals with inputs and outputs and its basic tasks are to identify, measure, value and compare the costs and consequences of alternative courses of action (Drummond et al. 2005, pp 9-10). In addition to the evaluation of costs, a full economic evaluation answers the efficiency questions as well. Analyses in which costs are measured as monetary units and consequences as natural effects or physical units and the latter identified as a single effect of interest, are referred to as cost-effectiveness analyses. This may include a cost-minimization analysis, in which the consequence of two strategies is equivalent and a comparison of costs is made and expressed as cost per patient. The existing and new strategies can further be assessed by an incremental analysis, in which the difference in costs is compared with the difference in consequences. One strategy is said to dominate another if it is more effective and less costly (Drummond et al. 2005, pp 11-13, 126).

A decision tree is a graphic representation, which begins with a decision and shows how possible choices relate to the possible outcomes (Walker 2001). Using decision analytic modelling in economic evaluation provides many advantages. It provides a framework which reflects the possible outcomes that individuals of interest may experience and the potential impacts of different strategies on outcomes. It provides an estimate of expected costs and effects for the strategies being compared based on available data. Moreover, it facilitates the analysis of uncertainty related to the evaluation (Drummond et al. 2005, pp 277-278). Economic evaluation can be performed from various viewpoints such as the patient, the hospital or the health maintenance organization.

The methodological and parametric uncertainty of economic evaluation is assessed by the sensitivity analysis, in which the estimates of key variables or assumptions are altered to examine what impact they have on the study results. Moreover, the threshold values for key parameters that would cause the strategy to be too costly or not cost-effective can be defined. Variation of uncertain parameters one at a time poses a risk that interactions between variables may not be noticed, but these interactions can be assessed by two-way or three-way sensitivity analyses (O´Brien et al. 1994).

5.2. Economic analyses in TB diagnostics

The effectiveness and cost of molecular methods compared to conventional approaches in diagnosing TB and testing for drug susceptibility were assessed by decision tree analysis (Heymann et al. 1997). According to the model rapid diagnostic methods (NAA testing, identification with DNA probes and radiometric broth drug susceptibility testing) decreased the average time to correct diagnosis by 84 % compared to conventional methods (solid media cultures, biochemical testing for identification and conventional drug susceptibility testing). The authors concluded that tests with higher unit costs may lead to lower medical expenses when the sensitivity and speed of the tests are improved. However, the limitations of the study were that the combinations of methods compared do not correspond to those currently used, and costs associated with the isolation of smear-positive patients were not included. When only assay cost was determined, the cost implication of unrestricted use of a NAA testing in TB diagnostics was calculated to be approximately £ 42,500 per year (Shetty et al. 2000). The authors concluded that the investment in an additional test was considerable and should be guided by the local rate of positive *M. tuberculosis* cultures. Further evaluations of effectiveness or directed use of NAA testing was not assessed.

6. Aspects for clinical use of the NAA assays

Performance studies have revealed some subgroups in study populations which may cause uncertainty in interpreting NAA results. In the study by Querol and colleagues (1995) PCR was positive in ten patients with old residual TB lesions in chest radiographs. Active disease could not be shown during 1-year follow-up, and both smear and culture results remained negative. Similarly, as a side finding 11/24 (46 %) of patients with previous history of TB but with no active disease were found positive by the PCR test (Beige et al. 1995). Although PCR assay proved more sensitive than culture, Beige and his co-authors concluded that these false positive PCR results are problematic with respect to clinical specificity. The capability of a PCR test to detect nonviable *M. tuberculosis* DNA in a routine laboratory setting was shown by Loeffelholz and co-workers (1996).

Another group disrupting the performance estimates in the studies is those patients receiving antituberculous chemotherapy. Reischl and co-workers (1998) reported two NAA-positive but culture-negative specimens originating from two patients with a history of TB treatment of 4 and 6 months. In the study by Dalovisio and colleagues (1996) 27 out of 259 patients had received treatment for 0 to 82 days. One or more positive results were detected in all patients by the culture and in 21-23 out of 27 patients by the NAA test. Ichiyama and co-workers

(1996) found that conversion of NAA assay results of 47 patients under therapy occurred only after conversion of culture was achieved. The complexity of interpreting the NAA results can be demonstrated in the study with a large material of 7,722 respiratory specimens (Levidiotou et al. 2003). A total of 254 specimens grew *M. tuberculosis* in culture. Fifteen culture-negative and PCR-positive specimens from patients receiving therapy (3 weeks to 6 months) were interpreted as true positive, whereas 15 PCR-positive specimens from patients who had completed treatment 2-3 months earlier were classified as false positive. Thus, it is obvious that patients under chemotherapy should not be included in studies investigating the diagnostic accuracy of the NAA tests. Nevertheless, these studies underlined the importance of a patient's clinical history and the need for more accurate assessment of the role of NAA tests in monitoring treatment response as well as their capability of detecting mycobacterial DNA in specimens of patients with past TB.

As important as the extensive investigations of the laboratory performance of NAA tests are evaluations of the usefulness of NAA assays in clinical practice and their impact on patient management. Conditions in laboratories as well as epidemiological situations and local prevalence of NTM differ between countries. The NAA technique requires skilled personnel and expensive equipment and reagents. Moreover, clinicians using the NAA assay should be aware of its competence in their clinical laboratory. Therefore, in addition to performance studies, implementing the NAA assay in routine practice demands clinical evaluations of its most appropriate use, and cost-effective analysis of the testing procedure (Schluger and Rom 1995, Heifets 2000).

Aims of the study

The aim of the present study was to evaluate the clinical usefulness of the commercial nucleic acid amplification assay in the diagnosis and management of tuberculosis patients in a low-incidence area. The detailed aims were:

1. To estimate the specimen- and patient-based competence of the NAA assay in detecting *M. tuberculosis* complex from sputum specimens of patients with suspicion of TB.

2. To study the performance of the NAA test in sputum specimens of patients with a history of active TB and residual lung lesions.

3. To evaluate the usefulness of NAA testing in monitoring the treatment response of patients with pulmonary TB.

4. To determine factors affecting the cost-effectiveness of using the NAA test in clinical practice.

5. To assess the practicability of the NAA assay in a real clinical setting and its optimal use in the management of patients with suspicion of TB.

Subjects and methods

7. Subjects, study design and ethics

The study series was based on five separate populations. The characteristics of the subjects are summarised in Table 7.

Table 7. *Characteristics of the study subjects and the tests used in Studies I-V.*

Study	Study population	Subjects (n)	Sex (M/F)	Mean age [range] (years)	Specimens (n)	Specimen type[1]	Tests used
I	Patients with TB[2] suspicion	151	103/48	67 [1-93]	324	S	Smear, culture, NAA test[3]
II	War veterans with past TB	25	25/0	76 [72-83]	152	S	Smear, culture, NAA test
	Subjects with recent TB	19	15/4	60 [43-74]	116	S	Smear, culture, NAA test
	Controls	34	30/4	73 [57-84]	190	S	Smear, culture, NAA test
III	TB patients	15	11/4	57 [28-81]	416	S	Smear, culture, NAA tests[4]
IV	Patients with TB suspicion	1219[5]	ND[6]	ND	ND	S	Decision tree model
V	Patients with TB suspicion	176	103/73	63 [0.2-93]	366	R + E	Smear, culture, NAA test

[1]S, sputum; R, respiratory including sputum; E, extrapulmonary; [2]TB, tuberculosis; [3]Cobas Amplicor PCR; [4]Cobas Amplicor PCR and AMTD2; [5]The mean number of the patients yearly tested for TB in two University Hospital Districts; [6]ND, Not determined.

7.1. Study I

Altogether 169 patients from the clinics of Tampere University Hospital and Pirkanmaa Hospital District were included to investigate the laboratory performance of the NAA assay in detecting *M. tuberculosis* complex from sputum specimens. Patients were clinically suspected of having pulmonary TB, and 1-4 sputum samples from each patient were collected for smear, culture and PCR testing. Fifteen patients with cultures growing NTM were excluded from the final comparison. Moreover, three patients with smear-positive but culture- and PCR-negative specimens were not included due to previous or subsequent detection of NTM and no active disease during the follow-up. Finally 151 patients were left in the NAA evaluation.

The results of the NAA assay were compared to the culture, which was considered the gold standard. In the event of discrepant results patient records were reviewed and clinical diagnosis was used as the final reference standard. The Se, Sp, PPV and NPV of the NAA test were determined firstly per specimen, and secondly per patient. In patient-based evaluation, the effect of multiple specimen collection on the sensitivity of NAA assay was also assessed.

7.2. Study II

Two different groups were studied to find whether false positive NAA results are detected in a population with a history of tuberculosis and subsequent residual lesions in lungs. Originally all 62 war veterans in the Pirkanmaa Hospital District registered in the State Treasury were invited to participate in the study. They had pulmonary tuberculosis in the 1940s or 1950s. Moreover, 46 subjects who had pulmonary TB with cavitary lesions during the period 1983-1993 were recruited. Altogether 44 veterans (group 1) and 24 former TB patients (group 2) attended the study protocol. The inclusion criteria included residual tuberculous lesions on chest radiograph and ability to expectorate sputum. Under the criteria 24 persons were excluded, leaving 25 veterans in group 1 and 19 subjects in group 2. In addition 34 patients with chronic obstructive lung disease from Pirkanmaa Hospital District participated in the study as controls. They were adjusted by age and sex and had neither history of TB nor residual TB scars in chest radiographs.

Patient records with regard to past tuberculosis were reviewed, and all subjects were asked about the history of their past disease and current symptoms with a standard questionnaire. Chest radiographs were taken and interpreted by an experienced chest radiologist. Additionally two sets of three sputum samples were collected on consecutive days at least two weeks apart and tested by smear, culture and the PCR test. The results of the tests were compared to clinical pictures and the information of patient records.

7.3. Study III

To evaluate the clinical utility of the NAA test in monitoring the efficacy of TB treatment, 23 consecutive newly diagnosed smear-positive TB patients admitted to the pulmonary clinics of Tampere University Hospital and Turku University Central Hospital were recruited for the study. The diagnosis was based on positive culture of *M. tuberculosis* and both clinical symptoms and findings of a chest radiograph consistent with TB. Those who declined to participate, were unable to expectorate sputum in spite of sputum induction, or died during follow-up were excluded from the final evaluation. The remaining fifteen patients completed the study. The treatment was carried out according to current guidelines and included INH,

RIF and PZA for the first 2 months, followed by INH and RIF for 4-8 months. Additionally, EMB and/or SM were given to five patients. No resistance was detected in isolated *M. tuberculosis* strains.

Clinicians were blind to the NAA results during the treatment of the patients. Patients´ compliance with INH therapy was monitored by monthly measures of urine INH. Two to three sputum specimens were collected on admission, at 1 and 2 weeks after the initiation of the chemotherapy, and thereafter monthly up to 6 months and at the conclusion of treatment if this was longer than 6 months. Additionally one set of specimens was collected after chemotherapy if patients could expectorate sputum. Specimens were tested by smear, culture, PCR and AMTD2, and the results were compared with each other and the clinical outcome.

7.4. Study IV

The aim was to compare the cost-effectiveness of two different laboratory strategies for diagnosing pulmonary tuberculosis: the conventional NOPCR strategy based on smear and culture tests, and the +PCR strategy including PCR test in addition to smear and culture. The study was performed with a decision tree model, which shows paths from the initial suspicion of TB to the eventual outcomes. The baseline population and the probabilities of the model were based on the retrospective 2-year patient data of Pirkanmaa and Varsinais-Suomi Hospital Districts. To avoid the impact of yearly fluctuations in low-incidence areas, combined annual data of the two districts were used, resulting in a baseline population of 1,219 with 27 (2.2 %) culture-proven TB cases and 35 (2.8 %) patients with NTM. The proportion of smear-positive patients was 3.1 %. The performance values of the NAA test applied in the model were obtained from Study I. All use of healthcare resources prior to confirmation of correct diagnosis by culture or the NAA test was calculated according to prevailing unit costs in the two hospital districts. It was assumed that the NAA test was performed twice a week providing results in 4 days, and the average time for a positive culture result was 2 weeks. Effectiveness was measured by the probability of correct treatment and isolation decisions.

7.5. Study V

The practicability of the NAA assay and its impact on management of patients with suspicion of TB was retrospectively analysed from laboratory and archive material of a 2-year period (Study V). The Pirkanmaa Hospital District implemented a strategy in which first 1-3 smear-positive specimens of the patient were automatically tested by the NAA test; moreover, it was applied to other specimens at clinicians' requests. In addition to patients tested by the NAA test, all smear-negative culture-confirmed TB patients not tested by NAA were included in

the evaluation. Three patients were excluded due to incomplete data and one because of a reaction to BCG vaccination, leaving 176 patients for the final evaluation.

The smear, culture and NAA results of diagnostic specimens were collected from laboratory records and separately compiled PCR lists. Patient records were reviewed to collect data on clinical symptoms, all diagnostic investigations, isolations and chemotherapy. The effect of NAA testing on clinical management was evaluated by comparing the dates of NAA test results with the dates of treatment initiation, and reviewing the clinical history of patients and treatment decisions recorded by the clinicians. The Se, Sp, PPV and NPV for the NAA assay were presented as per patient. Further, the actual delay of NAA results and use of the NAA test by practitioners were evaluated.

7.6. Ethics

The protocols of Studies I-V were approved by the ethics committee of Tampere University Hospital. In addition, approval for Studies III and IV was obtained from the ethics committee of Turku University Central Hospital. Subjects in Studies II and III gave written informed consent; and written permission was obtained from the veterans (Study II) to review the medical records in the State Treasury.

8. Methods

8.1. Specimen collection

Sputum specimens were collected according to the laboratory instructions of Tampere University Hospital. Study subjects were requested to expectorate a sputum sample into a plastic cup with a screw lid on three consecutive mornings. Cups were placed in a small plastic bag and stored in the refrigerator until transferred to the laboratory. Specimens from Turku University Central Hospital were cooled and transported to Tampere University Hospital in containers. Acceptable sample volume was 2-5 ml of sputum, and those specimens containing saliva were excluded. All respiratory and extrapulmonary specimens for NAA and culture testing were sent to the laboratory in sterile unbreakable cups or containers. In exception to that, for culture testing pleural and synovial fluid samples (2-20 ml) were collected in tubes containing anticoagulant, and blood and marrow specimens were inoculated directly into the BACTEC bottles.

8.2. Smear and culture tests

Specimens were digested and decontaminated by the NALC-NaOH method as described in Study I. All specimens were stained with the auramine fluorescent stain and viewed by fluorescence microscopy. Positive slides were confirmed by Ziehl-Neelsen stain and the light microscopy. Part of the decontaminated sediment (0.5 ml) was injected into a BACTEC 12B bottle (Panta Plus, Becton Dickinson Diagnostic Instrument Systems, USA) (Studies I-III), and later the BACTEC MGIT 960 liquid media (Becton Dickinson, Sparks, USA) was used (Study V). BACTEC bottles were read twice a week; MGIT included continuous reading. Part of the initial specimen (0.1 ml) was also injected into Löwenstein-Jensen tubes, which were incubated at 37 °C and checked weekly for positive culture. If no growth was detected in 6 weeks a negative result was reported. After that incubation was continued 4 more weeks for slowly growing mycobacteria such as *M. malmoense.* All positive cultures were tested by Ziehl-Neelsen staining to confirm the presence of acid fast bacilli, which were further identified by AccuProbe RNA-DNA hybridization (Gen-Probe Inc.). The rest of the initial specimen was either used for immediate NAA testing or divided into 2-3 parts and stored at –20 °C for later testing.

8.3. NAA testing

The Cobas Amplicor MTB test (Roche Ltd.) was performed according to the manufacturer's instructions (Studies I-III, V). The prepared specimen (50 μl) was transferred to the amplification tube, which contained the master mix including primers for *M. tuberculosis,* nucleotides, DNA polymerase and the internal control. Carryover contamination was prevented by AmpErase. Positive and negative controls were performed in each run according to the manufacturer´s instructions. Specimens and controls were amplified in the built-in thermocycler. Amplicons were detected automatically with target-specific DNA probes and the optical density at 660 nm was measured by the built-in spectrophotometer. An absorbance value ≥ 0.35 was interpreted as positive. If the internal control indicated presence of inhibitory substances, the specimen was prepared again and analysed both undiluted and diluted (1:10).

The RNA amplification was carried out with the AMTD2 test (Gen-Probe, USA) according to the instructions supplied by the manufacturer (Study III). A volume of 450 μl of each pretreated specimen was added to lysing tubes and rRNA was released from the target cells by sonication at room temperature. Part (25 μl) of the lysate was transferred to the amplification tubes containing amplification reagent and mineral oil. The tubes were briefly heated up to 95 °C to denature the target nucleic acid and to inactivate infectious agents. An enzyme reagent mixture was then added and amplification was accomplished during incubation at 42 °C for 1 hour. Amplification was terminated by the termination reagent. Detection of amplicons was

performed by a hybridization assay with acridinium ester-labelled DNA probes. Emitted light was measured by the luminometer. The test protocol did not include internal control. Each run contained positive and negative controls.

8.4. Interpretation of the test results

In patient-based evaluations the result of the test (smear, culture, NAA) was interpreted as positive if any of the 1-3 test results was positive, whereas the result was considered negative when all results of the concerned test were negative.

8.5. Statistical methods

Sensitivity, specificity, positive and negative predictive values were calculated to assess the diagnostic performance of the NAA assay compared to culture (Studies I, V). Performance values were separately calculated for smear-positive and -negative specimens (Study I) and patients (Studies I, V). The combination of culture and clinical data was used as a reference standard with discrepant results. The 95 % confidence intervals were calculated with the standard normal distribution formula to quantify the random error. Normality of the distribution of continuous variables was studied using the Kolmogorov-Smirnov test. The statistical comparisons in Study III were performed with the nonparametric Wilcoxon´s matched-pairs signed ranks test. Differences between various smear groups were tested by the Mann-Whitney test and between smear groups for impact of the NAA test by Fisher's exact test (Study V). A p-value of < 0.05 was considered statistically significant. SPSS for Windows (version 12.0, Chigaco IL, USA) were used for the statistical calculations. Calculations of the Study IV were carried out using a computer-based decision tree model built on DATA 3.5 (TreeAge Software, Inc., Williamstown, MA, USA).

Results

9. Diagnostic performance of the NAA assay (Studies I, II, V)

The capability of the NAA assay to detect *M. tuberculosis* from diagnostic samples was assessed from two points of interest: Firstly, a laboratory approach analysing specimen-based values, and secondly a clinical approach evaluating the accuracy of the NAA test to detect patients with tuberculosis. The performance values are summarized in Table 8.

With sputum specimens (N=324) the NAA assay reached a sensitivity of 83 % and specificity of 99 %. When one or two specimens were tested per patient, the sensitivity was 82 %, while by testing three specimens per patient it increased to 91 %, supporting the examination of 3 specimens per patient (Study I). In smear-positive specimens the sensitivity of the NAA assay was 90 %, and from the patient point of view all smear-positive TB patients (56) were detected by the NAA test (Studies I, V). The sensitivity of the NAA test to detect smear-negative TB disease (16 patients) was 75 % and 29 % in Studies I and V respectively, supporting the claim that smear-negative TB cannot be firmly excluded by negative NAA results.

Table 8. *Specimen- and patient-based results of the nucleic acid amplification (NAA) assay for detecting Mycobacterium tuberculosis complex from clinical samples.*

Type of evaluation	Study	Number/ Specimen type[1]	Smear +/ - TB[2]	Sensitivity [95 % CI[3]]	Specificity [95 % CI]	PPV[4] [95 % CI]	NPV[5] [95 % CI]	Sensitivity	
								Smear + [95 % CI]	Smear - [95 % CI]
			(n/n)	(%)	(%)	(%)	(%)	(%)	(%)
Specimens	I	324/S	51/25	83 [73-91]	99 [98-100]	97 [89-100]	95 [92-98]	90 [79-97]	68 [47-85]
Patients	I	69[6]/S	14/8	91 [71-99]	98 [89-100]	95 [76-100]	96 [86-99]	100 ND[7]	75 ND
Patients	V	148[8]/R+E	42/8	88 [76-96]	100 [96-100]	100 [92-100]	93 [86-98]	100 [92-100]	29 [4-71]

[1]S, sputum specimens; R, respiratory specimens including sputum; E, extrapulmonary specimens; [2]TB, tuberculosis; [3]CI, confidence intervals; [4]PPV, positive predictive value; [5]NPV, negative predictive value; [6]Three specimens per patient tested; [7]ND, not determined; [8]One to three specimens per patient tested.

In Study V one to three specimens per patient were tested reflecting the real-life situation, and 9 out of 50 (18 %) TB patients were extra pulmonary cases. The sensitivity was as high as 88 %, but was partly explained by the high proportion (84 %) of smear-positive TB patients.

Altogether 37 patients (Studies I, II, V) had NTM detected in culture testing. The species were *M. avium, M. gordonae, M. fortuitum and M. malmoense*. All these specimens tested negative in the NAA test. The capability of the NAA assay to differentiate between *M. tuberculosis* and NTM was shown in the clinical study (V), in which 14 smear-positive patients with NTM were negative in NAA testing.

Only two specimens from one patient were falsely positive in the NAA test in those three Studies (I, II, V). These specimens originated from a patient with previous TB and no active disease in the follow-up. On the other hand, in Study II, which concentrated especially on investigating patients with past TB and pulmonary scars, no false positive results were found either in 44 study subjects or 34 controls. Hence, the NAA assay resulted in good specificity and positive predictive values (Table 8). Inhibition of amplification was detected in altogether 8 out of 1,411 specimens yielding an inhibition rate of 0.6 % (Studies I-III, V).

10. NAA tests in monitoring treatment response (Study III)

Two different commercial amplification tests, the DNA amplification test (Cobas Amplicor PCR, Roche, Switzerland) and the rRNA amplification assay (AMTD2, Gen-Probe, USA) were evaluated for the feasibility of monitoring treatment response in 15 pulmonary TB patients (Study III). Altogether 416 specimens were collected during and after treatment. From the start of chemotherapy the mean time for smear, culture, PCR and AMTD2 to remain positive was 47, 68, 110 and 160 days respectively. The AMTD2 remained positive longer than the PCR in 60 % of patients. Further, four patients were positive in the NAA test on completion of successful treatment, but no association was found between the late positive results of the NAA tests and radiographic extent of TB. Subsequent specimens converted to negative within 2.5-12 months, and no relapses were detected during a follow-up of up to 3 years. From a clinical point of view, the performance of the PCR and AMTD2 tests during treatment did not significantly differ from each other.

11. Cost-effectiveness of the NAA testing (Study IV)

The cost-effectiveness of the conventional and PCR strategies in testing patients with suspicion of TB was assessed by the decision tree model (Study IV). When the PCR test was applied to all specimens from suspected TB patients and performed twice a week, the +PCR (smear, culture and PCR) strategy was not found to be cost-saving compared to the NOPCR (smear and culture, no PCR) strategy. The incremental cost per patient was € 29.50 with the +PCR strategy. The sensitivity analysis revealed three significant factors influencing the cost of the +PCR strategy: the performance time and cost of the PCR assay and the proportion of smear-positive patients in the tested population. According to the threshold analysis, if the PCR test was performed daily in an ideal setting, or the cost of the PCR assay was < € 97, or the proportion of smear-positive patients was over 4.0 %, the +PCR strategy would reduce costs compared to the NOPCR strategy. Further, the two-way sensitivity analyses indicated that the less costly the PCR assay is, the smaller the proportion of smear-positive patients in the tested population can be for the +PCR strategy to be cost-saving and vice versa.

It was found that the highest costs in both strategies originated from isolation and treatment of smear-positive patients. Hence, applying the +PCR strategy to only smear-positive specimens was assessed. Rapid differentiation between *M. tuberculosis* and NTM by the PCR test resulted in savings in isolation and inpatient care costs in the model. Consequently, the +PCR smear-positive strategy cost € 95 less per patient and produced more correct treatment and isolation decisions than the conventional NOPCR strategy. The economic model was found robust to changes in the variable, which reflects the sensitivity of the PCR test for smear-positive TB cases. Moreover, no threshold value was detected for the variable expressing the sensitivity of the PCR test for smear-negative TB cases. This was explained by the small proportion (0.7 %) of smear-negative TB patients in the baseline population.

According to the analysis of the model data, routine application of the PCR assay to all specimens from suspected TB patients was not cost-saving, whereas applying PCR testing to smear-positive specimens dominated the NOPCR strategy in terms of costs as well as correct treatment and isolation decisions.

12. NAA testing in a real-life clinical setting (Study V)

The clinical performance of the NAA assay was assessed retrospectively from a compiled 2-year data corpus of the routine use of the NAA test in various clinics of Tampere University Hospital. To supplement the study material (148 patients), those smear-negative TB patients not tested by the NAA test (28 patients), were also included in the evaluation. The performance values are presented in Table 8. Only 2 out of 8 smear-negative TB patients were positive in the NAA test. Four culture-positive specimens (1 sputum, 3 extra pulmonary specimens) from four patients were falsely negative. Additionally, two specimens from two patients were negative by both culture and NAA tests, and the diagnosis was confirmed in cultures of other specimens from the same sites.

The NAA testing had an impact on the early management of 26 out of 148 (18 %) patients, of whom 22 (32 % of all smear-positive patients) were smear-positive and 4 (5 % of all smear-negative patients) smear-negative. The NAA test was positive in all 42 smear-positive TB patients, but it influenced the diagnosis and treatment initiation in only 5 patients, because the diagnosis and treatment decision of smear-positive TB patients were mostly (37 patients, 88 %) based on clinical picture and radiological findings. Moreover, diagnosis and need for contact investigation was confirmed by the NAA test in 9 (21 %) patients. In addition NAA testing was of benefit in excluding TB in 8 out of 26 smear-positive patients with NTM or other disease; and 5 out of 12 unnecessary isolations were discontinued due to negative NAA results.

Two clinically important findings were also detected in the study. In smear-positive TB patients the median NAA result delay was found to be 7 days (range 1-27 days), and chemotherapy was started on 36/42 smear-positive TB patients prior to receipt of the NAA results. Furthermore, it was found that only 8 out of 36 (22 %) smear-negative TB patients were tested by the NAA assay at clinicians´ request.

Discussion

13. Study subjects

The study included altogether 386 subjects from four separate study populations (Studies I-III, V), which are summarized in Table 7. In terms of tuberculosis, the subjects represented three different types of patients: patients with a suspicion of TB (85 %), patients with past TB (11 %) and patients who were receiving antituberculous chemotherapy (4 %). The proportion of the patients is balanced from the viewpoint selected for the study series, although the total number of study subjects is not very high (mainly in the laboratory study, Study I), compared to the other studies (Table 3). This naturally impairs generalisation of the results. However, it reflects the low incidence of TB in Finnish population and the local laboratory circumstances, which are challenged by the resources as well as limited number of tested specimens (Studies I, V). The other study populations (Studies II, III) were selected subgroups, which were recruited systematically either from an existing limited population (war veterans), from a certain time period (patients with past TB) or within a set time period (patients receiving TB treatment). The sample size of these studies is in line with other focused studies (Hellyer et al. 1996, Moore et al. 1996). The group of war veterans was quite unique in two ways; firstly, they represented patients who had received intensive TB treatments but not effective chemotherapy in the 1940´s to the 1960´s, and secondly, detailed documents of their past disease and treatments were available for investigation in the State Treasury.

The proportion of women in the study population was 34 %, which is comparable with the sex distribution (34-38 % in pulmonary and 42-47 % in all TB patients) in Finnish TB population during the period 1997-2001 (National Public Health Institute 2006b). The age of the study population ranged widely, from 0.2 years to 93 years, but the distribution was skewed. The mean ages of 63 years in Study V and 57 years in the treatment study (III) are close to the average age of Finnish TB patients, which was 61-65 years in 1999-2001 (National Public Health Institute 2006a). HIV testing was not included in the study protocol. According to the patient records, neither HIV-TB co-infections nor MDR-TB patients were detected in the study population. This could be explained by the rareness of these diseases in Finland. Nevertheless, no subanalysis based on single or few such cases could have been performed.

14. Methodological considerations

In the present study two commercial NAA tests were applied: the Cobas Amplicor PCR (Studies I-V) and the AMTD2 (Study III). Standardized protocols and reagents were used, and test

procedures were automated, hence the results of the tests are comparable to those of other studies using commercial NAA assays. The PCR assay had an internal control to detect inhibitors. In the laboratory the volume and quality of the sample was first checked. Specimen preparation was done in separated areas according to standardized protocols by 2-3 experienced laboratory technicians, and negative controls were run parallel to detect possible cross-contamination. NAA tests were run in another room. The high quality laboratory work in Tampere University Hospital is assured by continuous training of personnel and by regular participation in external quality control programs for culture and staining procedures. Smear testing is performed by the fluorochrome method and confirmed by carbolfuchsin staining. Moreover, standardized decontamination procedures are used and culture sample is injected into both liquid and solid media as recommended to increase the yield of mycobacteria (Tenover et al. 1993). Based on these facts, it can be assumed that the quality of the laboratory work and the methods used are of good and sufficient quality.

The present thesis included prospective (Studies I-III) and retrospective (Study V) studies as well as modelling of clinical practice based on clinical data and prior study results (Study IV). The studies can also be classified as performance assessment (Study I), descriptive (Studies II, V) and observational (Study III) works. In the world of science the value and importance of such studies are obviously not ranked as highly as randomised double-blinded studies and generalising of the results is more problematic. However, this type of approach was appropriate for the objectives and design of the present study series, and further, the low yearly incidence of TB sets limitations for patient recruitment. Concerning the blinding of the NAA results, it was used for the clinicians in Study III, whereas blinding in Study V would have impaired the evaluation of the influence of NAA testing on clinical practice.

Economic evaluation should ideally incorporate clinical data, costs and effectiveness. The more implications and interventions are covered, the more complicated the decision tree model would be. Therefore, decision tree models are always simplifications of reality and require assumptions. The value of the model depends on what perspective is used and which effects are measured. The model applied in the present study assessed the cost-effectiveness of the NAA assay in terms of hospital charges, which could eventually reflect the costs to be born by society. Moreover, avoiding unnecessary treatments and isolations is important both from the patient and health care perspective. The decision tree was based on decision and treatment paths, corresponding as closely as possible to clinical practice. The variations in TB and NTM incidences, costs and diagnostic practices were considered by using combined data of two Finnish University Hospital Districts. All clinically appropriate paths were included in the model and variations of key parameters were tested by the sensitivity analyses. The NAA test performance values used in the model were obtained from Study I, from the same setting the model was applied to. Hence, it may be assumed that the framework and methods used were appropriate, and the model represented a setting where the TB incidence is low and NTM are fairly frequently detected.

Some factors should be considered when analysing NAA results and comparing them to those of other studies. In addition to different study populations, the combination of specimen type and proportion of smear-positive specimens vary considerably between studies. The lack of an explicit gold standard for active tuberculosis is evident. The culture method has its weaknesses and clinical determination is not uniform in all countries. Moreover, the prevalence and incidence of TB and NTM as well as manifestations of active disease in various populations differ from each other. Different economic parameters such as health sector resources and valuing of input prices influence the cost-effectiveness of diagnostic techniques. These factors must be considered if the results are applied to other settings.

15. Evaluation of the results

15.1. Laboratory performance of the NAA assay

In the present study the sensitivity of the NAA assay in detecting *M. tuberculosis* complex from sputum specimens was 83 %. This is at the same level as in other studies evaluating different NAA assays such as the Cobas Amplicor (Reischl et al. 1998, Levidiotou et al. 2003), LCx (Viinanen et al. 2000) and the Probe Tec ET (Maugein et al. 2002). Sensitivities of over 90 % have also been reported (Gamboa et al. 1998, Coll et al. 2003), but frequently the relative proportion of smear-positive specimens in the study material has been high (Wang and Tay 1999, Scarparo et al. 2000, Piersimoni et al. 2002) or the population has included patients receiving antituberculous chemotherapy (Ichiyama et al. 1996).

For a diagnostic test sensitivity under 90 % is not optimal. Like other authors (Reischl et al. 1998, Levidiotou et al. 2003), we observed that the sensitivity is impaired by the modest performance of the NAA assay in smear-negative specimens (Studies I, V). This finding does not support routine use of this test with smear-negative specimens (Sarmiento et al. 2003). It is further proved by the result of the economic evaluation (Study IV), in which that particular strategy proved not cost-effective. Moreover, the sensitivity of the NAA test in smear-negative patients is not adequate enough to enable exclusion of TB by negative NAA results (Studies I, V). However, the specificity and positive predictive value of the NAA test were found to be high, in other words very few false-positive results were detected (Studies I, II, V). These results, and the finding of a sensitivity of 75 % in smear-negative patients, support the use of the NAA test for those smear-negative patients who are strongly suspected of having TB. Although the number of smear-negative TB patients in our study was small, this suggestion is in line with the conclusion in the meta-analysis evaluating PCR for diagnosis of smear-negative pulmonary tuberculosis (Sarmiento et al. 2003).

In view of the results presented, another more evident target group for NAA testing would be smear-positive patients. The NAA assay was shown to be highly sensitive and cost-effective

when used on smear-positive specimens or patients (Studies I, IV). In addition, NAA testing distinguished *M. tuberculosis* accurately from nontuberculous mycobacteria (Studies I, II, V). These results are in accordance with other studies (Scarparo et al. 2000, Coll et al. 2003, Michos et al. 2006).

The hypothesis of the possibility to detect mycobacterial DNA by the NAA test from the sputum specimens of war veterans with past TB was considered important and clinically relevant (Study II), because in Finland we have a large population of senior citizens with lung scars due to past pulmonary TB. It was also supported by studies performed in the 1960´s. Asp (1962) observed in his investigation that, in contrast to cultivable bacteria, the duration of tuberculostatic (INH, PAS and SM) chemotherapy did not influence the occurrence of acid-fast bacteria in histological lung specimens. Sutinen (1968) reported further that AFB was detected in the resected lungs of TB patients, whose median duration of disease was 5 years and median duration of sputum negativity 6 months prior to surgery. Seventy percent of the cavitary lesions and 25-33 % of the fibrous lung and pleura tissues were found positive by auramine-rhodamine staining. Moreover, no significant correlation between tuberculostatic chemotherapy and the amounts of AFB in tissues could be demonstrated. However, we eventually found no false positive results with the NAA test in either war veterans or subjects with more recent TB. This might be explained by the time elapsing since active disease and subsequent healing; DNA material might have been scanty in fibrous lesions and possibly further not connected to bronchial airways.

It was also shown that a rapid result service for NAA testing was difficult to maintain at the regional level laboratory (Study V). It is evident that testing about 150-200 specimens a year with the NAA test is not a sufficient volume to maintain effective operation. Preparing a few or single specimens and running NAA tests at infrequent intervals is time-consuming and inefficient from the laboratory perspective. Hence, centralizing NAA testing to the national level in a low-incidence area seems rational. But even at the national level laboratory, and regardless of the NAA method used, careful assessment must be made to ensure frequent testing and rapid reporting of the results to the clinics.

15.2. NAA tests in monitoring treatment response

When the NAA tests were assessed for monitoring treatment response (Study III), their results were found to remain positive clearly longer than of culture. This finding is similar to those reported in other studies either evaluating in-house (Hellyer et al. 1996, Yuen et al. 1997) or commercial tests (Moore et al. 1996, Iinuma et al. 1998). In the present study 4 out of 15 initially smear-positive patients were positive in the NAA test at the end of chemotherapy. However, no correlation with clinical or radiological findings was detected in contrast to earlier studies, where PCR persistence was associated to the radiological extent of the disease (Iinuma et al. 1998) as well as previous drug treatment and multi-drug resistance (Yuen et al. 1997).

This might be explained by the heterogeneous study populations, since these studies included more severe cases, and a substantial part of the PCR converters were initially smear-negative. Furthermore, PZA was not used in the treatment protocol (Iinuma et al. 1998), which might have impaired the treatment and prolonged the mycobacterial DNA persistence in patients with advanced radiological lesions.

The finding of the AMTD2 results remaining positive longer than those of the PCR was slightly unexpected, while rRNA was known to decay more rapidly than DNA in vitro after cell death (van der Vliet et al. 1994). The AMTD2 persistence also argued against the study in which all AMTD results of initially smear-positive patients converted to negative before the end of therapy (Moore et al. 1996). However, that study used the AMTD1 test with a smaller sample volume and no internal control, which may have impaired the sensitivity of the test. The AMTD2 assay in turn uses a larger volume than the Cobas Amplicor PCR, and targets rRNA, which is present in thousands of copies in mycobacterial cells compared to 10-20 copies of the target DNA, and therefore might theoretically be more sensitive.

According to the results of Study III, it is suggested that commercial DNA and rRNA amplification assays are not appropriate for monitoring the treatment response of patients with smear-positive pulmonary tuberculosis. This is based on the fact that all patients had drug-sensitive disease and were successfully treated, and no relapses were detected. Yet in this population the results of the NAA assays were found to be inconsistent, and 27 % of successfully treated patients were positive in the NAA test at the end of chemotherapy. Further, NAA results did not correlate with the radiological extent of the disease, and no clinical difference was found between the performance of commercial DNA and rRNA amplification assays. One could argue that the inconsistency of the NAA results may be related to the small population size. However, these results are consistent with studies that evaluated quantitative NAA tests in monitoring treatment efficacy of TB patients. A study applying a semi-quantitative PCR method demonstrated that patients with moderate disease reached the baseline for negative PCR results after 6-8 months of treatment and patients with extensive disease within a year (Thomsen et al. 1999). Moreover, the decline of *M. tuberculosis* DNA was not found to correlate with the disappearance of cultivable mycobacteria (Desjardin et al. 1998), and further, the clearing of mycobacterial IS6110 DNA and 16SrRNA during chemotherapy was shown to be slow and not to reflect the bactericidal effect (Desjardin et al. 1999).

15.3. Cost-effectiveness of the NAA assay

Scarcity of resources compels health care providers to carefully evaluate which diagnostic strategies are worth implementing and maintaining in clinical practice. As mentioned earlier, performing NAA tests is costly; in terms of TB diagnostics the NAA test would be an additive test rather than a substitute for smear and culture tests. Therefore, key elements influencing the economic efficiency of the new strategy must be determined, and the particular benefit of using the NAA assay could be evaluated by means of effectiveness and savings resulting from

rapid and correct diagnosis of TB. The present study (IV) demonstrated that routine testing of all TB suspects by the NAA test was not cost-effective. This is in line with previous findings (D`Amato and Miller 1995, Shetty et al. 2000). As shown by the threshold analysis (Study IV), reducing the price of the NAA test would improve its cost-effectiveness. However, considering the cost development of NAA assays during the last ten years, substantial cost reduction seems unlikely, and if it happened, it would not resolve the problem of the technical expertise and high quality work needed to perform NAA tests.

The other method to reduce the input costs is to apply NAA testing to those subgroups which benefit the most. From the patient perspective needless investigations as well as unnecessary isolations and consequent contact investigations certainly influence patient comfort. These are also of economic importance, since up to 80 % of total hospital charges can arise from the isolation of infectious patients (Wurtz and White 1999). Hence, testing smear-positive patients with the NAA assay would be appropriate. This study demonstrated that NAA testing was cost-effective when applied to smear-positive specimens (3 specimens per patient); it produced cost-savings by reducing inpatient care days and unnecessary isolations in patients with NTM (Study IV). This was in accordance with the findings of D`Amato and Miller (1995). Moreover, Michos and co-workers (2006) reported that applying NAA testing to smear-positive specimens was cost-effective when test costs were compared to the number of correct diagnoses. In contrast to our results, Dowdy et al. (2003) reported in their economic modelling study that routine implementation of the NAA assay in smear-positive specimens was not shown to be cost-effective although only one smear-positive specimen per patient was tested, and the NAA test was performed 6 days a week. This can, however, be explained by the different perspective (health care system versus patient or hospital perspective) and costs used in that study. One might also criticize the slightly unrealistic baseline assumptions in the model: namely the availability of the NAA results in 24 hours at a regional level with 14 smear-positive patients detected annually, and excluding NTM with only one negative NAA result.

One should be aware that economic modelling does not specifically evaluate the importance of costs and consequences among different patient populations, and cost-effectiveness is not equivalent to clinical and epidemiological benefits. An example of this is that in our study (IV) the economic analysis was robust to the sensitivity of the PCR test for smear-positive cases because misdiagnosed patients did not create additional costs prior to culture confirmation. However, in clinical practice and epidemiologically misdiagnosed infectious TB patients would have a substantial effect on TB control.

15.4. The role of NAA testing in clinical practice

Although the results of the laboratory study (I) indicated, that the NAA assay is a competitive test in the diagnosis of smear-positive tuberculosis, the clinical study (V) revealed somewhat different results. Namely it was shown that most diagnoses and decisions on treatment initiation

of the smear-positive TB cases were based on the patient's history, clinical symptoms and smear results. This is consistent with the findings of Piersimoni et al. (2005). NAA testing was thus of value in those cases in whom differentiation between TB, NTM or other disease was difficult. This is also the patient group in which the cost savings are achieved when correct diagnoses are made (Study IV).

To our knowledge this was the first study (V) to assess the NAA test delay in a real clinical setting. The median result delay was shown to be 7 days, which clearly exceeds the median of 1 day reported for smear (Pascopella et al. 2004), and inevitably impairs the benefit gained from the rapidity of the NAA test. With smear-positive TB cases, NAA testing led rather to case confirmation than case detection and supported initiation of contact screening. Further, in smear-positive patients with diseases other than TB, negative NAA results reduced unnecessary isolation days and TB medications. Centralizing of NAA testing may reduce the result delay, but requires careful planning at the national level as discussed earlier.

In the present study (V) NAA testing was requested by the clinicians for only 22 % of smear-negative TB patients, and the test seemed to be used rather as a screening test for smear-negative patients. However, as mentioned above, negative NAA test does not with certainty exclude TB. The target is thus to detect patients with smear-negative TB, and more efficient selection of tested patients is needed. Pre-test estimates have been shown to accurately discriminate between patients with high and low risk of TB, but overestimation of TB is possible in the intermediate risk group (Lim et al. 2000). The overall diagnostic accuracy of the PCR test was reported to be > 95 % when respiratory specialists selected the patients and interpreted the test results (Lim et al. 2003). The Study V was retrospective and descriptive, and hence no systematic training in indications for NAA testing and interpretation of the results was arranged. Our results, however, underlined the importance of this kind of training, and thereafter studies of its impact on clinical practicability of the NAA assay are warranted.

16. General aspects

The development of NAA assays has progressed considerably since their introduction to clinical practice in the 1990´s. However, to date the optimistic expectations concerning the use of these tests in diagnosing tuberculosis have not been fulfilled. Molecular techniques have been firmly established in species identification, susceptibility testing and genotyping of the strains, but the exact and widely accepted role of NAA testing in the diagnosis and management of TB patients is still undefined (Figure 1). There are several factors contributing to this. The NAA assay cannot replace smear and culture tests. Therefore its use in addition to conventional tests induces incremental costs. Moreover, early diagnosis of smear-negative patients in clinical practice is particularly difficult and a tool to improve the diagnosis is needed. However, the performance of NAA assays is rather modest in this particular patient group due to random expectoration of mycobacteria in sputum, uneven distribution of bacteria in samples and the

paucibacillary nature of the extrapulmonary specimens. Hence, the performance of the NAA assay in smear-negative specimens is considerably affected by the nature of *M. tuberculosis* bacteria and the disease itself. It may be argued, however, that from a clinical point of view the performance of the NAA test in smear-positive patients is at least as important as in smear-negative patients. Namely, the diagnosis of smear-positive tuberculosis leads to other major acts such as the initiation of complicated chemotherapy, placing the patient in isolation and starting a contact investigation. Therefore, early confirmation of the correct diagnosis is essential, especially in areas where nontuberculous mycobacteria are frequently detected in clinical specimens.

Applying NAA tests in clinical practice can also be contemplated from the perspective of society or the country. The dilemma is that rich industrialized countries have laboratory resources of high quality, but the TB incidence is low, leading to a small number of specimens tested. Developing countries have plenty of TB patients and specimens, but the resources and technical means are inadequate to maintain NAA testing. Hence, an optimal context for NAA testing might be a country with both gross domestic product per capita and TB incidence at or above the medium level. However, a good health care system is also important including fluent cooperation between primary and secondary as well as private and public health care providers.

This study series was performed in a low-incidence area with a high quality laboratory service and well-educated clinicians, a setting similar to many industrialized countries. The results of the good sensitivity and specificity of the NAA test in smear-positive patients and the ability to differentiate *M. tuberculosis* from NTM confirmed earlier findings (Studies I, II, V). Moreover, the futility of the NAA test in monitoring the treatment of 15 pulmonary TB patients was demonstrated, and was in line with previous studies (Study III). New aspects in using the NAA assay were also identified. No false positive results were detected in two different patient populations with lesions of past tuberculosis (Study II). Further, the clinical study (V) revealed that testing of all diagnostic smear-positive specimens was not necessary since the diagnosis of TB is mostly based on smear and clinical judgment. Therefore, NAA testing can be offered to those patients, in whom distinction between *M. tuberculosis* and NTM or other disease is needed. An important new finding was the PCR result delay, which considerably impaired the clinical usefulness of the test (Study V). It concurrently revealed the vulnerability of the test procedure at the regional laboratory level.

Finally, the present dissertation clarifies the indications for using the NAA assay in TB diagnostics in the population described above, and reveals some important limitations, which must be considered in routine use. New and enhanced versions of molecular tests are being developed at an accelerating speed. One should be aware, however, that a comprehensive evaluation from the laboratory and clinical as well as from the economical perspective has to be carried out to assess the definitive suitability of each new test in the particular health care context.

Summary and conclusions

Over many decades the microbiological diagnosis of tuberculosis has been based on unspecific smear microscopy and sensitive but rather slow culture techniques. Despite their weaknesses, both methods have an essential role in the diagnostics. Therefore an additive test, which would allow rapid and accurate detection of *M. tuberculosis,* is greatly needed. Molecular methods have substantially enhanced strain identification, susceptibility testing and epidemiological monitoring. However, the exact role of NAA testing in the diagnosis of TB has not been firmly established.

The present study evaluated the usefulness of the commercial NAA test in the diagnosis and management of tuberculosis patients, focusing particularly on pulmonary tuberculosis. Altogether 386 subjects and 34 controls with an average age of 66 years (range 0.2-93 years) and sex ratio (M/F) of 298/140 were investigated by smear, culture and NAA tests. In addition, an analysis of the cost-effectiveness of the NAA assay was performed using a decision tree model.

The main conclusions drawn from the results of Studies I-V are as follows:

1. The overall performance of the NAA assay in detecting *M. tuberculosis* complex from sputum specimens assessed by Se, Sp, PPV and NPV was good compared to culture, and no notable inhibition of amplification was found. In patient-based evaluation the sensitivity further increased when three sputum specimens per patient were tested. The competence of the NAA test was demonstrated especially in smear-positive specimens and patients, whereas the Se for smear-negative specimens was found to be modest. However, the PPV in smear-negative specimens was high, indicating that a positive NAA result is significant in terms of active disease, but if clinical assessment reveals a discrepancy, other alternatives have to be evaluated (Study I).

2. The NAA test gave no false positive results in smear-negative subjects who had scars and residual lesions in chest radiographs consistent with past TB. This was shown in two different study groups: those who decades earlier had received inefficient drug therapy and surgical treatment, and those who had completed a course of effective TB chemotherapy. Nor were any positive results with the NAA test detected in the control group, which included patients with chronic obstructive pulmonary disease. These findings suggest that a positive NAA test result would primarily be an indicator of an active TB disease (Study II).

3. The NAA results during effective treatment of compliant patients with drug-susceptible tuberculosis were found to be inconsistent, and positive results were detected in 4 out of 15

patients at the completion of the chemotherapy. Further, no clinically significant difference between commercial DNA and rRNA amplification assays was detected. It is therefore suggested that these qualitative tests cannot be used for monitoring the treatment response of pulmonary TB patients (Study III).

4. According to the decision tree model based on clinical data and prevailing hospital charges, testing of all patients with TB suspicion by the NAA test in addition to conventional tests was not cost-saving. The main reasons were the cost and long result delay of the test as well as the low relative proportion of smear-positive patients in the tested population. However, the PCR strategy (smear, culture and the PCR test) was cost-effective compared to the conventional strategy (smear and culture), when the NAA test was applied only to the specimens of smear-positive patients. It reduced costs and resulted more frequently in correct treatment decisions and isolations (Study IV).

5. During 2-year routine use of the NAA assay in clinical practice, most treatment decisions of smear-positive TB cases were based on clinical judgment and smear results. NAA testing was found to be beneficial in one third of the smear-positive patients: that was, confirming the TB diagnosis and the need for contact investigation, or excluding TB in those patients who had NTM or other disease, which in turn led to discontinuation of unnecessary isolations and treatments. The median result delay of the NAA test was one week, indicating that an effective result service is difficult to maintain at the regional laboratory level with small number of specimens. The selection of patients for NAA testing proved problematic for the clinicians, since only less than one fourth of the smear-negative TB patients were tested by the NAA test during the study period. (Study V).

In conclusion, routine use of the NAA test in either smear-positive or smear-negative specimens is not recommended in a low-incidence area. The NAA assay is recommended to be used in smear-positive patients, particularly when distinguishing between TB and NTM or other disease is problematic. It may also improve the diagnosis of TB in smear-negative patients with strong suspicion of TB. A positive NAA test result is indicative of active disease, whereas negative NAA results do not definitely exclude TB.

It is evident that further development of more sensitive and less costly commercial NAA assays for diagnosing of smear-negative TB is warranted. However, in terms of either smear-positive or smear-negative patients, the impact of any new NAA test on TB diagnostics is dependent on the clinical speed of the test and efficient selection of patients for testing. Thus, the challenge from the laboratory perspective is to organize centralized specimen processing with a rapid results service; and from the clinical perspective to train the personnel in the proper use and interpretation of the test and improve the clinical skills of the practitioners using the tests. This study also addresses the importance of laboratory, clinical and economic evaluation in the particular health care context where the new test is to be implemented and sustained.

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Iiris Rajalahti

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SHORT COMMUNICATION

Mycobacterium tuberculosis complex is not detected by DNA amplification assay in sputum specimens of patients with lung scars due to past pulmonary tuberculosis

I. Rajalahti,* P. Vuorinen,† R. Järvenpää,‡ M. M. Nieminen§

*** Department of Pulmonary Diseases, † Centre of Laboratory Medicine, and ‡ Department of Clinical Radiology, Tampere University Hospital, § Department of Respiratory Medicine, Medical School, Tampere, Finland**

SUMMARY

OBJECTIVE: To evaluate detection of false-positive sputum amplification assay results in former tuberculosis patients with residual pulmonary scars.

DESIGN: A total of 268 sputum specimens from 25 war veterans with tuberculosis during 1940–1959, without adequate chemotherapy, and 19 subjects effectively treated for cavitary tuberculosis during 1980–1993 were tested by smear, culture and DNA amplification, as were 34 controls with no history of tuberculosis or pulmonary scars.

RESULTS: No active tuberculosis cases were identified. All specimens were negative on DNA amplification and smear. Eight specimens from six subjects were positive on culture, revealing atypical mycobacteria.

CONCLUSION: No genetic *Mycobacterium tuberculosis* material in sputum specimens of subjects with residual lesions of pulmonary tuberculosis and no false-positive amplification results were detected.

KEY WORDS: tuberculosis scars; past tuberculosis; nucleic acid amplification; PCR

IN MOST low-prevalence countries, reactivation tuberculosis is an important manifestation of the disease in the native population. Refugees and asylum seekers from countries with high tuberculosis prevalence also frequently display residual tuberculosis lesions on chest X-ray. In an acute clinical setting, the radiographic distinction between past and present pulmonary tuberculosis is often difficult.

In Finland, the incidence of pulmonary tuberculosis was around 290 per 100 000 population during and after World War II. Under the Military Injuries Act, the State indemnified medical expenses for war veterans with tuberculosis, and detailed patient records of tuberculosis patients have been preserved in the archives of the State Treasury. These patients received treatment that was very scanty compared to modern practice, and consequently most have residual tuberculosis scars on chest X-ray.

Gene amplification assays are rapid, sensitive tools in detecting *Mycobacterium tuberculosis* strains in sputum specimens.[1–3] Previous studies have indicated that polymerase chain reaction (PCR) tests also amplify non-viable DNA material.[4,5] In addition, false-positive DNA amplification results have been notified in patients with a history of tuberculosis and residual lesions on chest X-ray.[6,7]

To determine the influence of old radiographic tuberculosis lesions on the results of DNA amplification assay (Cobas Amplicor Mycobacterium Tuberculosis Test [MTB] PCR, Roche, Switzerland), we collected sputum specimens from 25 war veterans who had had tuberculosis without adequate chemotherapy and 19 individuals with a history of cavitary tuberculosis treated successfully with chemotherapy. Specimens were tested by smear, culture and Amplicor PCR.

MATERIALS AND METHODS

Subjects

Based on the archives of the State Treasury, all 62 war veterans in the Pirkanmaa Hospital District (population 450 000) were invited to participate in the study. The veterans had originally had pulmonary tuberculosis in the 1940s or 1950s (Group 1). Forty-six people with past cavitary tuberculosis disease during the period 1980–1993 were also invited (Group 2). Forty-four (71%) veterans and 24 (52%) recent TB patients attended the study protocol.

Inclusion criteria consisted of residual fibrotic tuberculosis infiltrations on chest X-ray and the ability to expectorate sputum. A total of 24 persons did not fulfil the inclusion criteria, and 25 subjects from Group 1 and 19 subjects from Group 2 completed the

Correspondence to: I Rajalahti, Department of Pulmonary Diseases, Tampere University Hospital, PO Box 2000, 33521 Tampere, Finland. Fax: (+358) 3-247 3012. e-mail: iiris.rajalahti@nic.fi

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final evaluation. The control group consisted of 34 patients (adjusted by sex and age) from the Pirkanmaa Hospital District who had chronic obstructive lung disease and no history of tuberculosis or residual scars on chest X-ray.

Study design

All subjects were interviewed about the history of their past tuberculosis and current symptoms by a qualified respiratory nurse using a standard questionnaire. Chest X-rays were taken within a month of the interview and interpreted by an experienced chest radiologist. Each subject also provided two sets of three sputum specimens on consecutive days at least 2 weeks apart. The study subjects received no antibiotics during sputum collection. Specimens were tested for the presence of *M. tuberculosis* by acid-fast smear, culture and Amplicor PCR at the Centre of Laboratory Medicine, Tampere University Hospital. The results were compared with each other and correlated with clinical pictures and patient records.

The study was approved by the joint Ethics Committee of the Pirkanmaa Hospital District and Tampere University Hospital.

Clinical specimens

A direct smear was first prepared at the laboratory. All specimens were screened using auramine fluorescent stain, and those positive were confirmed by Ziehl-Neelsen stain. Specimens for BACTEC culture and Amplicor PCR were digested and decontaminated by the N-acetyl-L-cysteine-NaOH (NALC-NaOH) method.[8] Part of the decontaminated sediment (0.5 ml) from each specimen was used to inoculate a BACTEC 12B bottle supplemented with PANTA PLUS (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD), and the rest was divided into three parts and stored at −20°C until Amplicor PCR was performed in batches within 2 weeks.

Culture identification

Mycobacterial cultures were incubated at 37°C for 6 weeks. BACTEC bottles were read twice a week with BACTEC 460 apparatus (Becton Dickinson). A growth index of >100 was considered positive. Positive cultures were stained with Ziehl-Neelsen to confirm the presence of acid-fast bacilli, which were further identified by Accu-Probe RNA-DNA hybridisation. Accu-Probe was used according to the manufacturer's instructions, and as described earlier.[3]

Amplicor PCR

Amplicor PCR was carried out according to the manufacturer's instructions and as previously described.[2] The internal control of amplification was introduced into each amplification reaction and was co-amplified and detected with the possible target DNA from the clinical specimen.

RESULTS

There were 25 male war veterans in Group 1 and four females and 15 males in Group 2. The mean ages in Groups 1 and 2 were respectively 76 (range 72–83) and 60 years (range 43–74). Eighteen veterans were initially tested only by smear, and 15 proved positive. Accordingly, part of the tuberculosis diagnoses in Group 1 were based on clinical pictures and X-ray results. Thirteen (52%) veterans had relapsed and two received modern chemotherapy. Fifteen subjects (79%) from Group 2 were positive on smear, and one subject had relapsed.

Past tuberculosis disease on X-ray was mainly extensive, and current residual radiographic lesions varied from scanty infiltrations to chest deformations (Table 1). Excluding the four thoracoplasty patients, in Group 1 the current median thickening of the apical pleura was 17.5 mm (range 0.5–70.0) and the diameters of the cavitary infiltrations in three subjects were 10, 17 and 60 mm. In Group 2, the median diameter of past active tuberculosis cavitary infiltrations was 19 mm (range 12–33), and the diameters of the two current residual cavitary infiltrations in two subjects were 19 and 22 mm.

Of the 44 study subjects, 21 (48%) had predisposing factors for tuberculosis during the study, such as ongoing diseases (cancer, diabetes, silicosis), oral corticosteroid medication or at least three of six symptoms (cough, sputum, haemoptysis, weight loss, night sweats and increased body temperature). Eighteen (53%) of the controls were receiving frequent or constant oral corticosteroid medication, and 14 (41%) reported at least three of the above symptoms. However, after accurate clinical, radiological and microbiological evaluation, no active tuberculosis cases were identified.

Table 1 Radiographic extent of past active tuberculosis (TB) disease and of current residual lesions in Groups 1 and 2

	Group 1 *n* = 25 *n* (%)	Group 2 *n* = 19 *n* (%)
Past active TB		
Both lungs involved	20 (80)	12 (63)
Parenchymal infiltrations	25 (100)	19 (100)
Cavitary infiltration	9 (36)	17 (89)
Pleurisy	13 (52)	2 (11)
Mediastinal lymphadenopathy	1 (4)	1 (5)
Current residual lesions		
Both lungs involved	19 (76)	11 (58)
Fibrotic apical infiltrations	23 (92)	19 (100)
Cavitary infiltration	3 (12)	2 (11)
Pleural adhesions and calcifications	20 (80)	7 (37)
Thorax deformities	6 (24)	0 (0)
Bronchiectasies	5 (20)	5 (26)

Table 2 Results of smear, culture and Amplicor PCR assay in Groups 1 and 2 and in the control group

	Sputum specimens *n*	Smear positive/ negative *n*	Culture positive/ negative *n*	PCR* positive/ negative *n*
Group 1 (*n* = 25)	152	0/152	4†/148	0/152
Group 2 (*n* = 19)	116	0/116	0/116	0/116
Controls (*n* = 34)	190	0/190	4‡/186	0/190

* Inhibition was not detected.
† *M. avium* × 1, *M. gordonae* × 3.
‡ *M. fortuitum* × 4.
PCR = polymerase chain reaction.

A total of 268 sputum specimens from the 44 study subjects (mean six per subject) and 190 specimens from the 34 controls (mean six per subject) were evaluated. None of the sputum specimens were positive on smear, whereas eight specimens from six subjects were positive on culture (Table 2). All of the positive culture results originated from atypical mycobacteria (*M. avium, M. fortuitum* and *M. gordonae*). All of the Amplicor PCR results were negative in both study groups and in the control group. Inhibition was not detected.

DISCUSSION

We have previously reported good sensitivity and specificity of Amplicor PCR in detecting *M. tuberculosis* from sputum specimens in the Finnish population, indicating the role of the PCR test in confirmation of new, active, especially smear-positive, tuberculosis.[2,3] In order to clarify the performance of Amplicor PCR test in situations of past pulmonary tuberculosis, we evaluated two groups with different histories of tuberculosis. The subjects of both groups had suffered moderate or severe tuberculosis and had residual scarring in their lungs. Theoretically, anaerobic conditions with insufficient chemotherapy probably favour the formation of latent infection with dormant *M. tuberculosis* in residual lesions, and fibrotic cavitary infiltrations might be potential reservoirs for residual mycobacterial DNA.[9]

Two sets of three sputum specimens were collected at least 2 weeks apart to exclude incidental excretion of mycobacterial material. None of the tested specimens yielded *M. tuberculosis* on culture, and active tuberculosis disease was not diagnosed. Atypical mycobacteria were cultivated in eight specimens, reflecting a common situation in patients with chronic lung lesions. All sputum specimens with atypical mycobacteria were negative on Amplicor PCR, as expected. Negative smear results were also predicted due to the low sensitivity of smear. Our negative Amplicor PCR results strongly argue against the findings of Querol et al., who reported positive in-house PCR results in 10 of 44 patients with old residual lesions and no active disease or relapse on follow-up.[10] As they assumed, one explanation for these positive PCR results might have been contamination. Our data are in accordance with those of Viinanen et al., who obtained negative results by ligase chain reaction (LCx) from patients with residual chest radiograph changes.[11]

We found no false-positive results on Amplicor PCR among either inadequately treated or effectively cured tuberculosis patients with residual pulmonary scars. Negative Amplicor PCR results indicate that possible dormant mycobacteria or DNA material in residual lesions were too scanty to be detected by the PCR test or that this material was not in contact with the bronchial airways.

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RÉSUMÉ

OBJECTIF : Evaluer la détection de résultats faussement positifs dans les tests d'amplification des expectorations chez d'anciens patients tuberculeux porteurs de cicatrices pulmonaires résiduelles.

SCHÉMA : Ont été testés par frottis, culture et amplification de l'ADN, 268 échantillons d'expectoration provenant de 25 vétérans de la guerre ayant été atteints de tuberculose pendant la période 1940–1959 sans chimiothérapie adéquate et de 19 sujets traités de façon efficace pour une tuberculose cavitaire pendant la période 1980–1993. Trente-quatre sujets-contrôle sans antécédents de tuberculose ou sans cicatrice pulmonaire ont été examinés de la même manière.

RÉSULTATS : On n'a identifié aucun cas de tuberculose active. Tous les échantillons se sont avérés négatifs au frottis et à l'amplification de l'ADN. Chez six sujets, huit échantillons ont été positifs à la culture, qui a mis en évidence des mycobactéries atypiques.

CONCLUSION : On n'a détecté aucun matériel génétique de *Mycobacterium tuberculosis* dans les échantillons d'expectoration de sujets porteurs de lésions résiduelles de tuberculose pulmonaire, ni aucun résultat faussement positif de l'amplification.

RESUMEN

OBJETIVO : Evaluar la detección de resultados falsos positivos en los tests de amplificación en la expectoración de pacientes tuberculosos antiguos con cicatrices pulmonares residuales.

DISEÑO : Se examinaron con baciloscopia, cultivo y amplificación de ADN 268 muestras de esputo provenientes de 25 veteranos de la guerra con tuberculosis entre 1940 y 1959 sin quimioterapia adecuada y 19 sujetos tratados eficazmente por tuberculosis cavitaria durante el período 1980–1993, así como 34 controles sin historia de tuberculosis o de cicatrices pulmonares.

RESULTADOS : No se identificaron casos de tuberculosis activa. Todas las muestras fueron negativas a la amplificación de ADN y a la baciloscopia. Ocho muestras provenientes de seis sujetos fueron positivas al cultivo, que reveló la presencia de micobacterias atípicas.

CONCLUSIÓN : No se detectó material genético de *Mycobacterium tuberculosis* en las muestras de esputo de los sujetos con lesiones residuales de tuberculosis pulmonar ni resultados falsos positivos de la amplificación.