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Drug-Resistant *Mycobacterium tuberculosis* in Estonia

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ABSTRACT

Tuberculosis (TB) infection and disease patterns among different populations are extremely heterogeneous. This thesis explores mainly by microbiological methods the epidemiology of TB in Estonia.

Through the work high rates of drug resistance were found. The first countrywide study carried out in 1994 ascertained that drug resistant TB, and particularly multidrug-resistant TB (MDR-TB) is a serious problem for Estonia. Initial resistance to one or more of the drugs tested was 28%, with 10% being initially multidrug-resistant. Molecular typing with IS6110 RFLP has revealed that 29% of Estonian *M. tuberculosis* isolates belonged to the genetically closely related group of strains with a predominant IS6110 banding pattern. This isolates were classified by spoligotyping as Beijing genotype strains, widely found in Asia. The majority (87.5%) of all multidrug-resistant isolates and two thirds (67.2%) of all isolates with any drug resistance belonged to Beijing genotype family.

The incidence of TB among health care workers (HCW) in Estonia was 1.5 to 3 times higher (mean 91/100 000/year) than in the general population. In a chest hospital the incidence was 30 to 90 times higher. The highest rate was observed among physicians. In addition, this work shows that nosocomial isolates of *M. tuberculosis* can often be MDR after MDR-TB becomes more common in the general population. More than one third (38%) of all *M. tuberculosis* isolates obtained from HCW were multidrug-resistant.

The investigation of means by which drug resistance evolves among drug-susceptible *M. tuberculosis* strains during antiTB treatment revealed that initially drug-susceptible *M. tuberculosis* does not always evolve drug resistance despite highly irregular and prolonged therapy. Yet, the remained susceptibility of *M. tuberculosis* does not grant treatment success. When advanced method of molecular typing is not employed, exogenous re-infection with drug resistant *M. tuberculosis* may be misinterpreted as creation of drug resistance.

This work also encompass of our experience and knowledge on testing drug susceptibility of MDR *M. tuberculosis* isolates to second-line and alternative drugs. In Estonia, a standardized treatment regimen with up to 6 second-line drugs can be suggested for 2/3 of MDR-TB patients; for the remaining cases additional testing of an extended panel of drugs is required.

The MDR *M. tuberculosis* clinical isolates with unusual kanamycin-resistant but amikacin-susceptible phenotype were characterized by identification of mutations with-in the *rrs* gene. Mutation possible related to intermediate level of resistance to kanamycin, showing a thymine for cytosine substitution at the 16S rRNA position 516, has not previously been associated with kanamycin resistance in *M. tuberculosis*. To date, genetic methods fail to detect all clinically relevant levels of drug resistance to aminoglycosides. Consequently it is important to test antimicrobial susceptibility of resistant clinical isolates of *M. tuberculosis* by culture.

Key words: tuberculosis, Estonia, *M. tuberculosis*, drug resistance, MDR-TB, Beijing genotype, cross-resistance, kanamycin, amikacin, rrs mutations, 16S rRNA, exposure, nosocomial transmission, mycobacterial persistence, re-infection, DST, second-line drugs, Bactec 460, DNA fingerprinting, spoligotyping.

LIST OF ORIGINAL PAPERS

This thesis is based on the following papers that will be referred to by their Roman numerals.

I. Krüüner A., Sillastu H., Danilovitsh M., Levina K., Svenson S.B., Källenius G., Hoffner S.E. Drug resistant tuberculosis in Estonia. The International Journal of Tuberculosis and Lung Diseases. 1998; 2:130-133.

II. Krüüner A., Hoffner S.E., Sillastu H., Danilovitsh M., Levina K., Svenson S.B., Ghebremichael S., Koivula T., Källenius G. Spread of drug resistant pulmonary tuberculosis in Estonia. Journal of Clinical Microbiology. 2001; 39:3339-3345.

III. Krüüner A., Danilovitsh M., Pehme L., Laisaar T., Hoffner S.E., Katila M.L. Tuberculosis as an occupational hazard for healthcare workers in Estonia. The International Journal of Tuberculosis and Lung Disease. 2001; 5:170-176.

IV. Krüüner A., Pehme L., Ghebremichael S., Koivula T., Hoffner S.E., Mikelsaar M. Use of molecular techniques to distinguish between treatment failure and exogenous reinfection with *Mycobacterium tuberculosis*. Clinical Infectious Disease. 2002; 35:146-155.

V. Krüüner A., Hoffner S.E. Susceptibility to second-line drugs in clinical isolates of multidrug-resistant *Mycobacterium tuberculosis* from Estonia. *Submitted for publication*.

VI. Krüüner A., Jureen P., Levina K., Ghebremichael S., Hoffner S.E. 16S rRNA mutations in kanamycin-resistant but amikacin-susceptible clinical *Mycobacterium tuberculosis* isolates. *Submitted for publication*.

CONTENTS

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LIST OF ABBREVIATIONS

AIDS	Acquired Immuno Deficiency Syndrome
AK	Amikacin
ATS	American Thoracic Society
ATP	Adenosine Triphosphate
CAP	Capreomycin
CDC	Centers for Disease Control
CIP	Ciprofloxacin
CLA	Clarithromycin
CLO	Clofazimine
DNA	Deoxyribonucleic Acid
DOT	Directly Observed Treatment
DR	Direct Repeat
EMB	Ethambutol
ENRL	Estonian National Reference Laboratory
ETH	Ethionamide
EuroTB	Surveillance of Tuberculosis in Europe
GI	Growth Index
GLC	Gas Liquid Chromatography
HCW	Health Care Workers
HIV	Human Immunodeficiency Virus
HPLC	High Pressure Liquid Chromatography
IC	Infection Control
INH	Isoniazid
IS	Insertion Sequence
IUATLD	The International Union Against Tuberculosis and Lung Disease
KM	Kanamycin
LAM	Lipoarabinomannan
L-J	Löwenstein-Jensen
MDR	Multidrug Resistant
MGIT	Mycobacterial Growth Indicator Tube
MIC	Minimal Inhibitory Concentration
PCR	Polymerase Chain Reaction
PGRS	Polymorphic Guanine-Cytosine-Rich Sequence
PZA	Pyrazinamide
RFLP	Restriction Fragment Length Polymorphism
RIF	Rifampicin
RNA	Ribonucleic Acid
SM	Streptomycin
SMI	Smittskyddsinstitutet (Swedish Institute for Infectious Disease Control)
SSCP	Single-Strand Conformation Polymorphism
TB	Tuberculosis
WHO	World Health Organization

INTRODUCTION

Tuberculosis (TB) is a chronic infectious disease that has afflicted humanity for over 35 000 years (Huges *et al.*, 2002), and its etiological agent, *Mycobacterium tuberculosis*, has accounted for more human deaths than any other pathogen (Cole, 1994). In industrialized countries, the effect of the disease on society was weakened by the radical improvements in living conditions that occurred in the middle of the 20th century, and further by the implementation of modern chemotherapy during the past 50 years.

However, during the past decade TB has begun to re-establish itself, and strains of *M. tuberculosis* resistant to one or more of the main anti-tuberculosis drugs are emerging. Drug resistance, in particular the treatment of patients with multidrug resistance, complicates TB treatment. Few effective drugs are available and therefore infection with drug resistant *M. tuberculosis* could give rise to a potentially untreatable form of the disease. Regrettably, levels of drug resistance in Europe and particularly in Eastern Europe have not been well described. In addition, few epidemiological studies have been performed to analyze the spread of drug resistant *M. tuberculosis* strains in large populations.

Currently, TB is a common infectious disease in Estonia. Following a steady decline in incidence from 417 per 100 000 population in 1953 to 25.8 per 100 000, in early 1992, incidence reports have shown a steady increase beginning in 1993, and reaching 46.8 per 100 000 by 2002. This increase was accompanied with the emergence of drug resistant TB forms. The first country-wide study carried out in 1994, in collaboration with the World Health Organization (WHO) and Swedish Institute of Infectious Disease Control (SMI), ascertained that drug resistant TB and particularly multidrug-resistant TB (MDR-TB) is a serious problem for Estonia. Initial resistance to one or more of the drugs tested was 28%, with 10% being initially multidrug-resistant (WHO/TB/97.229). Anti-tuberculosis treatment, not matching the WHO's global treatment strategy, has been linked with high MDR-TB rates in Estonia (Espinal *et al.*, 2001).

New perspectives have been obtained in the control of infectious diseases through the use of molecular methods. In recent years, a large number of DNA (deoxyribonucleic acid) fingerprinting methods have become available to type mycobacterial isolates (van Soolingen, 2001). Thusfar, these methods, used for strain typing, have not been utilized in Estonia to aid in understanding the epidemiology of TB. Moreover, these methods may lead to the recognition of well-defined genotype families within the *M. tuberculosis* complex. Furthermore, they allow detection and estimation of the transmission of particular *M. tuberculosis* clones within the population from a recent common ancestor (Kurepina *et al.*, 1998).

The modes of action for different anti-tuberculosis drugs and the mechanisms of resistance for *M. tuberculosis* have been studied by several researchers (Cole, 1994; Lipsitch and Levin, 1997). So far the complete mechanisms of action of most anti-tuberculosis drugs are not fully understood.

The risk of TB among healthcare workers (HCW) was substantial in the era prior to the introduction of antibiotics, but declined rapidly after 1950 due to decreasing TB incidence in the population and the advent of effective therapy (Menzies *et al.*, 1995). Over the past decade, two factors have profoundly altered views about the risk of TB

in HCW, the resurgence of TB and the emergence of MDR strains of TB (Menzies *et al.*, 1995).

However, there is a lack of thorough knowledge as to how *M. tuberculosis* is spreading in different societies and in different settings. Moreover, we do not know the extent of clonal spread of particular genotypes of *M. tuberculosis*, specifically the role of single clones such as the Beijing genotype strains, and their association with drug resistance. Further information is required to understand how different factors such as increased virulence, immunogenicity, transmissibility, survivability and fitness for causing infection favour the spread of MDR *M. tuberculosis* or how several conditions such as irregular chemotherapy, and poor compliance participate in the evolvement of drug resistance from drug susceptible *M. tuberculosis* strains.

LITERATURE REVIEW

HISTORICAL PERSPECTIVE OF TUBERCULOSIS

"A disease of the past returns to haunt the future" (Salyers and Whitt, 1994).

One may conclude from different historical sources (Corper, 1929; Webb, 1936; Keers, 1978), that the disease caused by a microbe, presumably identical or very similar to the currently recognized pathogen, *Mycobacterium tuberculosis*, has been extant in diverse human populations for many thousands of years. Indeed, by using a variety of sophisticated molecular biological indicators, Hughes et al. (Huges *et al.*, 2002) recently estimated that the tubercle bacillus is at least 35,000 years old. Today tuberculosis is reappearing in many countries as a public health crisis. Thus, if not an emerging disease, it is an important reemerging disease, and though ancient, it is not a disease of the past. A staggering 1.9 million around the globe die of tuberculosis each year – another 1.9 billion are infected with *M. tuberculosis* and are at risk for active disease (Dye *et al.*, 1999).

Modern understanding of etio-pathogenesis of tuberculosis

TB has been described since the time of Hippocrates, who referred to it as "phthisis", signifying the wasting character of the disease. Aristotle correctly determined its contagious nature, observing that, " the consumptive has around him a pernicious air that is disease-producing" (Webb, 1936).

Centuries of uncertainty passed before Robert Koch clarified the situation in Berlin in 1882. Koch concluded that, 1) the life cycle of tubercle bacilli entailed human-to-human transmission without external, natural reservoirs: that the microbes "are true and not occasional parasites"; 2) the portal of entry was the lungs, and the source of the microbes were patients with cavitary disease who expectorated immense numbers of bacilli; 3) "TB has so far been habitually considered to be a manifestation of social misery, and it has been hoped that an improvement in the latter would reduce the disease". Most of Koch's findings are still valid and were highly useful prior to the appearance and swift spread of the human immunodeficiency virus (HIV).

M. tuberculosis has adopted a pattern of peaceful co-existence with its human host in the form of a quiescent or dormant infection, establishing a massive reservoir of infected individuals – possibly including as much as one-third of the world´s population. But it can trigger progressive and deadly destruction of the lungs and act as the source for further dissemination. Moreover, it has forged a deadly partnership with the HIV- increased susceptibility to tuberculosis, and tuberculosis in turn accelerates the progression to AIDS (Young, 1998). HIV exerts a pronounced influence on the natural history of TB with higher rates of reactivation of disease, acute disease, increase of extrapulmonary TB and malabsorption of anti-tuberculosis medications (Sepkowitz *et al.*, 1995). The containment of these growing problems will be one of the great challenges in public health in the near future.

Mycobacteria were first described in the late 1800s with the discovery of the tubercle bacillus, originally named *Bacterium tuberculosis* (Zopf, 1883) and the leprosy bacillus, originally named Bacillus leprae (Hansen, 1880). The classification of mycobacteria began in 1896 when Lehmann and Neumann (Lehmann and Neumann,

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1896) proposed the genus *Mycobacterium* to include these species (renamed *Mycobacterium tuberculosis* and *Mycobacterium leprae*). The genus was placed in the *Mycobacteriaceae* family, *Actinomycetales* order and *Actinomycetes* class (Shinnick and Good, 1994).

The entire genome of the H37Rv strain of *M. tuberculosis* was sequenced in 1998 allowing significant advances in understanding the bacterium's pathogenesis. The genome is composed of 4,411,529 base pairs, which form an estimated >4,000 genes (Cole *et al.*, 1998; Camus *et al.*, 2002). These data have afforded new opportunities for the design of antimicrobial agents, vaccines, and other elements which can protectively modulate the immunopathogenesis of this pathogen (Young, 1998). Also, the genome comparison amongst different isolates and between species plays an important role in understanding the epidemiology and evolution of the mycobacteria (Young, 2002).

Development of resistance to anti-tuberculosis drugs

In 1943 Selman Waksman and his colleagues discovered streptomycin and, for the first time, provided a chemotherapeutical approach to TB treatment as an alternative to fresh air, diet and physical exercise. In 1944 streptomycin was first employed, with high efficacy, to treat TB patients (Ayvazian, 1993).

However, soon after the initiation of streptomycin therapy it was recognized that not all cases of TB could be cured, due to the emergence of resistant mutants (Mitchison, 1950). The powerful anti-tuberculosis activity of isoniazid was first observed in 1951. Similarly, shortly after the introduction of isoniazid, the first resistant strains were isolated from patients treated by isoniazid monotherapy (Buck and Schnitzer, 1952). The majority of cases of resistance involved resistance to isoniazid and streptomycin until rifampicin was introduced in 1967. Subsequent to its introduction, rifampicin has been prescribed widely, and resistance to it has also emerged (Mitchison and Nunn, 1986). Resistance to rifampicin has been frequently associated with its use in an inadequate regimen and with noncompliance (Goble *et al.*, 1993).

The simultaneous occurrence of resistance to rifampicin and isoniazid greatly reduces the prospects for successful chemotherapy (Mitchison and Nunn, 1986). Multidrug-resistance (MDR), i.e. resistance to at least isoniazid and rifampicin, is currently the most severe form of bacterial resistance in TB cases (Chaulet *et* al., 1996). However, several authors had pointed out that the global magnitude of drug resistance for *M. tuberculosis* has not been well described (Cohn *et al.*, 1997; Rüsch-Gerdes, 1999).

MYCOBACTERIA

Taxonomy

"Mycobacterium" is derived from the Greek words for fungus (*myces*) and small rod (*bakterion*). The "fungus" component of the name derives from the tendency of these microorganisms to spread diffusely over the surface of liquid medium similar to a fungal growth pattern. The genus *Mycobacterium* is the only genus in the family *Mycobacteriaceae*. The high guanine-plus-cytosine (G+C) content of the DNA of *Mycobacterium* species (62 to 70%) is similar to that of the other mycolic acid-producing bacteria, *Nocardia* (60 to 69%), *Rhodococcus* (59 to 69%), and

Corynebacterium (51 to 59%) (Murray *et al.*, 1999). There are at least three important criteria for classifying bacteria in the genus *Mycobacterium*: 1) acid- and alcohol-fastness, 2) presence of mycolic acids containing 60 to 90 carbons with C_{22} to C_{26} pyrolysis esters and, 3) G+C content of DNA in the range of 61 to 71 (Levy-Frebault and Portaels, 1992). Colony morphology, various biochemical tests, genomic studies, growth characteristics and requirements, are all currently in use to classify these bacteria into different species (Levy-Frebault and Portaels, 1992; Wayne *et al.*, 1996; Wayne and Kubica, 1986). At present, more than 100 species of mycobacteria have been validated and are listed on the internet at http://www.-sv.cict.fr/bacterio/mycobacterium.html with 30 known or suspected of being pathogenic to humans.

Mycobacterium tuberculosis complex

M. tuberculosis is the type representative of the family, conforming to the criteria/properties listed above. The *M. tuberculosis* complex, the cause of TB, is comprised of *M. tuberculosis*, *M. bovis*, *Mycobacterium africanum*, *Mycobacterium microti* and *Mycobacterium canettii*. The high degree of evolutionary conservation of *M. tuberculosis* complex strains is exemplified by their high degree of interstrain DNA homology (Imeada, 1985), the conservation of their 16S rRNA gene sequence (Kirschner *et al.*, 1993) and the similarity of their 16S to 23S ribosomal RNA (rDNA) intergenic spacer sequence (Frothingham *et al.*, 1994). Furthermore, repetitive DNA elements, such as the insertion sequence IS*6110* (van Soolingen *et al.*, 1991) and the direct repeat (DR) (Hermans *et al.*, 1991) have been found to be restricted to the *M. tuberculosis* complex.

The members of the *M. tuberculosis* complex can all cause TB in humans although *M. tuberculosis* is the most prevalent. The natural reservoir of *M. tuberculosis* and *M. africanum* is limited to humans and that of *M. microti* is mainly limited to small rodents (Wayne and Kubica, 1986; Kremer *et al.*, 1998). In contrast, the host range of *M. bovis* is very broad and this species causes disease among a wide range of wild and domestic animals as well as in humans (Thoen *et al.*, 1984; Brosch *et al.*, 2002). All members in the complex are slow-growing microbes with generation times ranging from 12 to 24 hours depending on environmental and microbial variables (Iseman, 2000).

One characteristic but not distinctive property of *M. tuberculosis* is the tendency to form "cords". The biochemical source of this phenomenon was identified by Bloch in 1950 and titled "cord factor" (Bloch, 1950). Cord factor, later identified as a highly unusual biological compound, trehalose 6,6'- dimycolate, was observed to be the cause high morbidity and lethality when injected serially into animal models (Bloch *et al.*, 1953). However, even today the role of this compound in the pathogenesis of TB is unresolved. Arguing against its central role in virulence, the compound is also found in other nonpathogenic mycobacteria.

Two lipopolysacharides, lipoarabinomannan (LAM) and lipomannan from the cell-wall structure of mycobacteria are involved in pathogenesis of mycobacterial disease (McNeil and Brennan, 1991). LAM is considered a mycobacterial virulence factor (Chan *et al.*, 1991) due to its ability to direct macrophage function (Sibley *et al.*, 1990, Chan *et al.*, 1991), inhibit the processing of mycobacterial peptides/proteins by antigen-presenting cells (Moreno *et al.*, 1988), and to induce tumor necrosis factor

production (Moreno *et al.*, 1989, Barnes *et al.*, 1990). Analysis has identified biochemical differences in the terminal structures of the LAM moieties from classically virulent and avirulent strains of *M. tuberculosis*, Erdmann and H37Ra, respectively (Chatterjee *et al.*, 1992). Ludwiczak and colleagues reported an alteration in the fine structure of lipoarabinomannan in a *pho*P knock out mutant of *M. tuberculosis* (Ludwiczak *et al.*, 2002). The *pho*P gene encodes part of a two-component response regulator and the knock out strain is attenuated in a muse model (Perez *et al.*, 2001).

CLINICAL LABORATORY AND TUBERCULOSIS

Laboratories serve a major role in the diagnosis and management of TB. For safety and proficiency reasons the concept of three levels (I, II, III) of laboratory services for mycobacterial diseases was proposed by Kubica (CDC-manual 1968) and accepted by the American Thoracic Society in 1983 (Levels of laboratory services for mycobacterial disease. American Review of Respiratory Disease, 1983, 128, 213). The concept of levels defines the parameters in laboratory services and proficiency monitoring (Isenberg, 1992). At present this concept is widely spread and generally accepted in most of the countries in Europe, including Estonia.

Microscopy

Smear examination is rapid, inexpensive, technically simple, and highly specific for acid-fast bacilli. Smear microscopy can not, however discriminate between *M. tuberculosis* and other mycobacteria and in addition lacks sensitivity as 5.000 to 10.000 bacteria/mL are needed for a positive result (Smithwick, 1976). The identification of smear positive patients is of major importance because only smear positive pulmonary TB patients are regarded as highly infectious to others. These cases are referred to as smear positive. Thus, microscopy remains the primary diagnosis in TB until novel techniques are completely developed (Iseman, 2000).

Cultivation and identification

Mycobacterial culture is the ultimate proof of mycobacterial infection and is often used as the reference method due to its high sensitivity and specificity (Schirm *et al.*, 1995; Walker, 2001). Also the cultivation of the etiological agent has been essential for species identification, drug susceptibility testing, and monitoring the response to therapy. The slow growth rate of *M. tuberculosis* (and most other mycobacterial pathogens) complicates the use of cultivation as a diagnostic technique.

Nevertheless, culture is considered as the reference method for detection of tubercle bacilli and other mycobacteria (Källenius *et al.*, 1994). Traditionally, egg- or agar-based solid media have been used for isolation of mycobacteria. On egg-based Löwenstein-Jensen media the cultivation times of at least 2-3 weeks before mycobacterial growth from clinical specimens can be obtained are common. More rapid growth is achieved by using liquid media, such as Middlebrook or Dubos broth. The introduction of the (Bactec TB-460) radiometric respiratory technique has reduced detection times to approximately 10 days (McClatchy *et al.*, 1983; Kirihara *et al.*, 1995). The Bactec 460 system uses a ^{14}C-labelled substrate with subsequent

detection of $^{14}CO_2$ produced in the Bactec bottles, reflecting the metabolic activity, in the form of growth index (GI) values.

If growth of mycobacteria is detected, the next task is to discriminate between *M. tuberculosis* complex and other mycobacteria. Traditionally, *M. tuberculosis* has been identified by the slow growth rate, accumulation of niacin, a positive nitrate reductase test and non-photochromogenicity. Alternatively, species identification may be carried out by analysis of mycolic and fatty acids by chromatographic methods such as gas liquid chromatography (GLC) or high pressure liquid chromatography (HPLC) (Butler and Kilburn, 1988). Today molecular biological methods such as DNA sequencing (Kirschner *et al.*, 1993; Kirschner and Bottger, 1998; Roth *et al.*, 1998), PCR-restriction fragment length polymorphism assays (Telenti *et al.*, 1993c; da Silva Rocha *et al.*, 1999; Roth *et al.*, 2000), and commercial tests such as the AccuProbe (Gen-Probe Inc., San Diego, Calif.) are available for identification of mycobacteria. Also, DNA strip technology, based on the reverse hybridization of PCR products to their complementary probes, has been applied to simultaneous detection and identification of mycobacteria. Currently, two DNA strip assays, INNO-LiPA MYCOBACTERIA (Innogenetics N.V., Ghent, Belgium) (LiPA) and GenoType Mykobakterien (Hain Lifescience GmbH, Nehren, Germany) (GenoType), are commercially available (Mäkinen *et al.*, 2002).

Drug susceptibility testing

Primary drugs- isoniazid, rifampicin, streptomycin, ethambutol and pyrazinamide.
After isolation of a *M. tuberculosis* complex strain from a TB patient, susceptibility testing is performed. The traditional methods using egg-base, agar-base or liquid media and follow one of the three different varieties of susceptibility testing, 1) the resistance ratio method, 2) the absolute concentration method or 3) the proportion method (Kent and Kubica, 1985; Canetti *et al.*, 1969; Siddiqi, 1995). The drug susceptibility testing of a *M. tuberculosis* strain isolated from sputum takes between 7 to 12 weeks (Heifets, 1991a).

Today there are several more rapid methods in use: Bactec, MGIT, ESP Myco, and MB/BacT, which are already developed or under development for commercial use (Heifets and Cangelosi, 1999). In all these systems, a modified 7H9 broth is used. An indirect susceptibility test has a turnaround time of 18 to 22 days for Bactec-460, 10 to 22 days for MGIT, and about 24 days for both the ESP and MB/BacT systems (Heifets and Cangelosi, 1999).

Jacobs *et al.*(1993) have performed *in vitro* drug susceptibility tests, employing firefly luciferase. The luciferase reporter phage is a useful tool for evaluating viability and involves infection of mycobacterial cells with a phage carrying the firefly luciferase gene. In the presence of adenosine triphosphate (ATP), found only in living organisms, luciferase produces light from its substrate, luciferin. If mycobacteria are treated with drugs prior to infection with the reporter phage, light is then produced only by viable or drug-resistant cells. Although simple, this method is not without drawbacks and appears more suited for screening novel anti-tuberculosis compounds than for evaluating drug susceptibility of clinical isolates (Cooksey *et al.*, 1993).

Second-line and alternative drugs. With the recent rise in multidrug-resistant strains of *M. tuberculosis* (WHO/19, 2000), there is an increasing demand for determining

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the *in vitro* susceptibilities of clinical isolates to antimicrobial agents other than those considered primary drugs. Unfortunately data on critical concentrations for classical second-line and other newer drugs have been largely fragmentary or lacking altogether. In 1999 Pfyffer *et al.* developed a basic protocol that defines appropriate critical concentrations for secondary drugs to allow reliable testing with both Bactec 12B and agar medium (Pfyffer *et al.*, 1999). Since 2001 the WHO guideline for drug susceptibility testing for second-line anti-tuberculosis drugs for Dots-plus (WHO/CDS/TB/2001.288) is available. However, this document do not include recommendations or standardized protocols for liquid media systems or other newer techniques.

For drug susceptibility testing the use of standardized methods with a quality assurance program including national and international proficiency testing is compulsory. In 1993 as a part of a long-term commitment, a collaboration was initiated by the Karolinska Institute and SMI in Stockholm, Sweden and the Tartu University Lung Hospital in Tartu, Estonia. The purpose of this collaboration was to standardize the drug susceptibility testing methods used in Estonia and to set up the program for proficiency testing. Since 1994 the TB laboratory at SMI has accomplished the international proficiency testing in Estonia.

In past decades, the only markers available to study the epidemiology of TB were drug susceptibility profiles and phage types (Crawford and Bates, 1985; Gruft *et al.*, 1984). The predictive value of phage typing to link TB cases is limited, because only a few phage types can be distinguish among *M. tuberculosis* isolates. In most areas, one phage type predominates amongst *M. tuberculosis* isolates; related and therefore the unrelated cases cannot be distinguished on this basis. The use of either method had serious limitations. The drug susceptibility profile of *M. tuberculosis* strains is a highly unstable feature, because strains frequently gain resistance to anti-tuberculosis drugs during treatment. Therefore, today these methods are not applicable for epidemiological typing of mycobacterial isolates.

However, recent years, a large number of molecular techniques have become available to type mycobacterial isolates for different purposes (van Soolingen, 2001; Moström *et al.*, 2002).

Molecular biological techniques

Molecular methods are increasingly being applied for three purposes in TB control these include 1) the detection of causative micro-organisms in clinical material, 2) rapid diagnosis of drug resistance and 3) the detection of transmission of *M. tuberculosis*. For the first task specific microbial genomic target sequences are amplified in polymerase chain reaction (PCR) or other DNA/RNA amplification assays (Roth *et al.*, 1997). Species identification may be achieved by utilizing semiconserved housekeeping genes such as the 16S rRNA gene (Busse *et al.*, 1996, Boddinghaus *et al.*, 1990). Molecular methods, which rely on the detection of mutations in the regions of gene(s) associated with drug action, may be applied for a rapid diagnosis of drug resistance (Telenti *et al.*, 1993a; Rossau *et al.*, 1997). To examine the transmission of TB, a high number of strain-specific genetic markers with different levels of discrimination, stability and reproducibility have been identified in the last decade (Saunders, 1995; Drobniewski *et al.*, 1994; Kremer *et al.*, 1999). Recently the methods for identifying genetic alterations in *M. tuberculosis* associated with pathogenicity of tuberculosis have been established. The genome

comparison techniques such as microarrays, genomic subtractive hybridization and two-dimensional bacterial genome display are powerful molecular tools designed to detect small deletions and insertions, point mutations, or genetic rearrangements possible modifying bacterial virulence (Salamon *et al.,* 2000; Schmidt *et al.,* 1998; Dullaghan *et al.,* 2002). To-date, the large variety of molecular techniques available, has not been applied to cases of *M. tuberculosis* infection in Estonia.

Rapid diagnosis of drug resistance

Our understanding of the mechanisms of resistance of *M. tuberculosis* to several anti-tuberculosis agents has improved following the development of genetic tools to study mycobateria, particularly due to the availability of powerful PCR techniques (Jacobs *et al.,* 1987; Saiki *et al.,* 1988). Several PCR-based strategies such as SSCP analysis (Telenti *et al.,* 1993b), heteroduplex analysis (Williams *et al.,* 1998); dideoxy fingerprinting (Felmlee *et al.,* 1995), an RNA/RNA duplex base pair-mismatch assay (Nash *et al.,* 1997), an rRNA/DNA-bioluminescence-labeled probe method (Cangelosi *et al.,* 1996), a reverse hybridization-based line probe assay (Rossau *et al.,* 1997) along with other strategies have been recently designed for the rapid detection of mutations associated with drug resistance.

Each of these molecular strategies has both advantages and disadvantages. The accuracy of these methods may vary for different drugs in addition certain assays are too complex and expensive for a wide clinical application. The line-probe assay strategy has the advantages of relatively reliable performance, and has been used to detect rifampicin resistance due to mutations in the *rpo*B gene (Ramaswamy and Musser, 1998). Such a rapid test would also detect the MDR *M. tuberculosis* strains since these are rifampicin and isoniazid resistant and almost all phenotypically rifampicin resistant strains are also resistant to isoniazid.

However, the failure to understand the complete mechanism of action of most anti-tuberculosis drugs may be associated with the presence of multiple genes associated with resistance to anti-tuberculosis drugs (Banerjee *et al.,* 1994, Mdluli *et al.,* 1998, Sherman *et al.,* 1996, Zhang *et al.,* 1992).

Detection of transmission of *M. tuberculosis*

In order to discriminate bacterial strains as much as possible, the best approach would be to sequence the whole genome of each strain. That would, however, be time consuming and not cost effective, and therefore only parts of the genome are examined (Moström *et al.,* 2002).

The use of molecular epidemiology to study transmission of *M. tuberculosis* is based on the principle that strains recently derived from a common ancestor exhibit a similar DNA fingerprint. The term "cluster" is used to indicate *M. tuberculosis* isolates with identical or highly similar DNA fingerprints, and also for defining the respective patients from whom these genetically related strains were isolated (van Soolingen, 2001). Thus, strains isolated from different patients, but belonging to the same cluster, may have a great probability of being epidemiologically linked, i.e. they may reflect recent transmission between the patients.

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Despite the genetic homogeneity in the *M. tuberculosis* complex, a high degree of DNA polymorphism is associated with repetitive DNA such as insertion sequence (IS) elements and short repetitive DNA sequences (Kremer *et al.*, 1999). Until recently, over 14 different kinds of insertion sequences (ISs) (Moström *et.al.*, 2002), IS*6110* (Thierry *et al.*, 1990), -*1081* (Collins and Stephens, 1991), - *1547* (Fang *et al.*, 1998), and the IS like element (Mariani *et al*, 1993), has been identified in *M. tuberculosis* complex strains genome. Of these genetic elements the IS*6110* has been studied most intensively. IS*6110* has been widely used as a genetic marker to differentiate clinical *M. tuberculosis* isolates for epidemiological investigations due to its apparent mobility and its common presence in, on average, large numbers of copies. IS*6110* has a size of 1,355-bp and is present only in the *M. tuberculosis* complex (Cave *et al.*, 1991).

The insertion sequence IS*6110* is present in different copy numbers (between 0 and 25 copies) in the *M. tuberculosis* complex and is integrated at various chromosomal sites. Restriction fragments based upon the IS*6110* element are highly polymorphic but stable enough for epidemiologic investigations (van Soolingen *et al.*, 1991; van Soolingen *et al.*, 1993). Recently in an inter-laboratory study, the current typing methods for *M. tuberculosis* isolates were evaluated with regard to reproducibility, discrimination, and specificity (Kremer *et al.*, 1999). This study concluded that for epidemiological investigations, strain differentiation by IS*6110* RFLP or mixed-linker PCR are the methods of choice. IS*6110* RFLP-based DNA fingerprinting has been used for the investigation of transmission of TB in hospitals (Edlin *et al.*, 1992), residential facilities for human immunodeficiency virus-infected patients (Coronado *et al.*, 1993), prisons (Greifinger *et al.*, 1992), laboratory cross-contamination (Bauer *et al.*, 1997), and in larger populations (Yang *et al.*, 1995; Yang *et al.*, 1998).

There are several genetic markers that can be used in addition to IS*6110* RFLP typing, especially when the strains of *M. tuberculosis* possess only one or two copies of IS*6110*. Relatively simple methods are spoligotyping (Kamerbeek *et al.*, 1997) and the genome-sequence-based fluorescent amplified-fragment length polymorphism analysis (FAFLP) typing (Goulding *et al.*, 2000). In addition polymorphic guanine-cytosine-rich sequence (PGRS) RFLP typing, is often used as a supplementary typing method (van Soolingen *et al.*, 1994; Cousins *et al.*, 1998; Burman *et al.*, 1997).

Today IS*6110*-RFLP is the reference standard for typing strains of *M. tuberculosis*. The method is considered to be the most discriminatory one and has therefore remained as the ultimate tool for identifying epidemiological clusters (Thierry *et al.*, 1990; McAdam *et al.*, 1990; van Embden *et al.*, 1993; van Soolingen *et al.*, 1993; Moström *et al.*, 2002).

However, the required stability of the genetic polymorphism, associated with different genetic markers, particularly with IS*6110* element, has been lately intensively discussed.

Stability of IS*6110* restriction fragment length polymorphism (RFLP)

The genetic polymorphism associated with the insertion sequence IS*6110* is of utmost importance in the epidemiology of TB and at the same time although it does have

shortcomings (van Soolingen, 2001). Strains need to change fast enough that non-epidemiologically related isolates are distinct, and yet slowly enough that isolates from related cases prove similar. Soon after the introduction of IS*6110* RFLP typing, transposition of the IS elements were recorded in the offspring of particular *M. tuberculosis* strains from epidemiologically related cases, exhibiting band shifts in the DNA fingerprints (van Soolingen *et al.*, 1991; Cave *et al.*, 1994; Godfrey and Stoker, 1992). This led to concern regarding the reliability of this method for epidemiological analysis of TB cases.

Recently, in two studies with a longer follow up period, IS*6110* RFLP patterns of isolates were compared for patients with sequential positive cultures during different intervals (Yeh *et al.*, 1998; de Boer *et al.*, 1999). In a Dutch-based RFLP study, using survival analysis, the half-life of IS*6110* RFLP was 3-4 years and in a San Francisco-based study the results were similar at 3.2 years. This implies that, on average, half of the strains exhibit a band shift in their IS*6110* RFLP pattern during a 3-4 year period.

This interval seems to be suitable for distinguishing epidemiologically related and unrelated isolates and therefore supports the use of IS*6110* typing in epidemiological studies with recent transmission of TB (van Soolingen, 2001). Furthermore, *in vitro* and *in vivo* studies have demonstrated the stability of the fingerprint pattern even when emerging drug resistance has been observed (van Soolingen *et al.*, 1991; Cave *et al.*, 1994; Niemann *et al.*, 1999).

Hence, the IS element is applicable for the detection of transmissions of TB in large populations. However, studies are required on the fate of *M. tuberculosis* strains in large populations from countries such as Estonia, which have an increase of TB, and MDR-TB is emerging. Moreover, studies on serial isolates obtained from patients with long TB episodes are needed to elucidate the kinetics of IS elements.

TUBERCULOSIS EPIDEMIOLOGY

Global

Approximately one-third of the world population, or 1.8 billion people, have been infected with *M. tuberculosis*. Active disease develops in 8 to 10 million people per year, and TB is responsible for 3 million deaths per year, making it the leading lethal infection in the world (Sepkowitz *et al.*, 1995). Projections made in late 1990s indicate that the incidence of all forms of TB may increase from a total of 8.8 million cases annually by 1995, to 10.2 million cases by 2000, and further to 11.9 million by 2005 (Styblo and Raviglione, 1997).

In 1998 TB case notification rates for the top 23 countries in TB cases in the world, per 100 000 of population, ranged from 81 to 597, with a median of 123 per 100 000. In industrial countries the notification rates were much lower, ranged between 5 and 35 per 100 000 population, with a median rate of 10 per 100 000 (WHO/CDS/TB/2000.275).

The global TB problem has been exacerbated by the pandemic of HIV infection with AIDS and the emergence of drug resistance, in particular multi-drug resistance. HIV infection renders a person infected by *M. tuberculosis* much more likely to develop

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overt TB, and in addition the evolution of the disease is considerably accelerated (Zumla and Grange, 1998).

Although, exact data on TB from Estonia's neighbouring regions such as Russia are not available, in 1997 approximately 25,000 Russians died from TB, and the new infection rate is one of the highest (estimated at 85,000 cases per year) in the world (Stern *et al.*, 1999).

In 1999 TB case notification rates for the Baltic countries were: 82.4 per 100 000 for Latvia; 78.8 for Lithuania, and 53.4 for Estonia. In contrast, Central and Western European countries like Hungary, Germany and Switzerland had notification rates of 38.8, 12.1 and 10.5, respectively and the downward trends were registered (EuroTB, 2001). Hopefully, similar trends may be expected in Baltic countries after further industrialization due to the raising of living standards, level of medical care and scientifically well-grounded interventions.

In Estonia

In Estonia, following a steady decline in incidence from 417 per 100 000 population in 1953 to 25.8 per 100 000 in the early 1992, the incidence reports showed a steady increase which started in 1993 and reached 46.8 per 100 000 in 2002. The incidence rates of new cases of active TB since 1970 to 2000 are shown in Figure 1.

Figure 1. Active TB incidence rates in 1970–2000
Incidence rate per 100 000 inhabitants
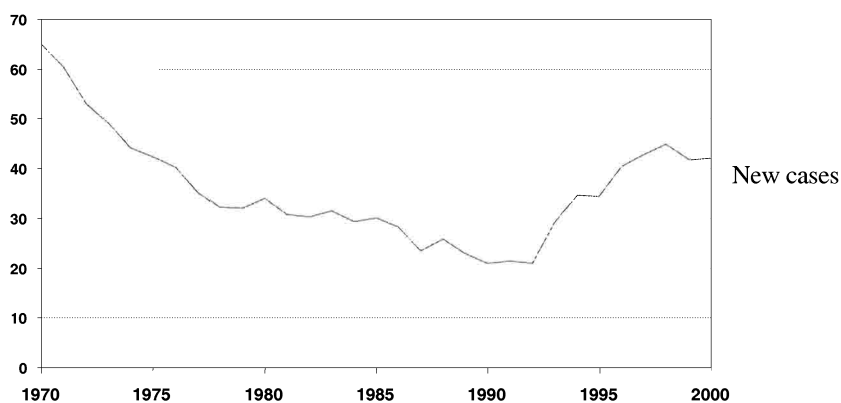
This unexpected step-by-step increase in morbidity has been accompanied by an increase in mortality from 5 to 8 per 100 000 in this period. In 2000, 69% of the new TB patients belonged to the age group of 20-54 years (Figure 2), with two thirds being men. Pulmonary TB comprised 91% of the cases, of which 73% were verified by smear or culture.

During the last decade the proportion of extrapulmonary TB has remained low (8 - 12%) (Statistical Yearbook of Estonia 2000).

Figure 2. TB age-specific incidence rate per 100 000 inhabitants, year 2000

Age-specific incidence rate
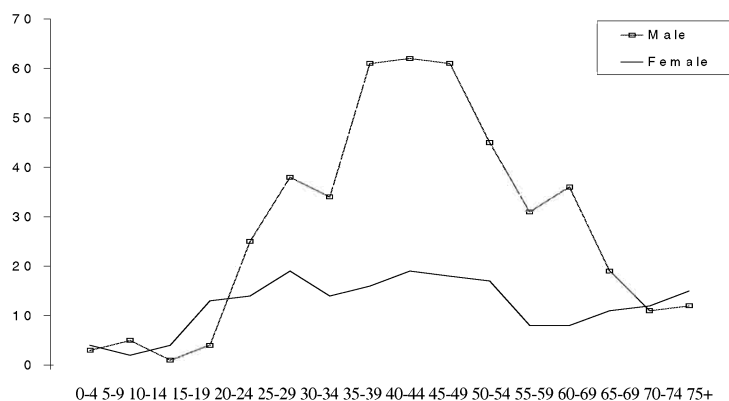
To date, the possibilities for linking the clinical, sociodemographic and microbiological data for individual TB patients have been very limited. A solution for this problem was to create an advanced and efficient TB register.

It was not known whether the increase in TB incidence rates in 1993 has been accompanied with the emergence of drug resistance. Prior to 1994 nation-wide data on drug resistance of *M. tuberculosis* was not collected for analysis. Furthermore, the drug susceptibility testing methods used in Estonia in that period was neither validated nor standardized preventing accurate assessment for the levels of drug-resistant TB.

Tuberculosis register for tuberculosis control

Prior to 1996 data on TB incidence in Estonia were collected and analyzed by the statistician at Kivimäe Hospital in Tallinn. A limited number of data sets were reported for all new TB cases including name, sex, age, site of disease, cavity presence, smear/culture positivity, county or city of residency and date of registration. Since 1996 the new electronic TB register was initiated and created by the same institution. Today, with the advice and contribution of international experts from EuroTB, NO-TB-BALTIC, WHO, IUATLD, Green-Light Committee and the National TB program team members, the register has attained a level of functionality and includes the spectrum of variables required for improving TB surveillance and epidemiologic investigations within the community.

The TB register may serve as an important tool in TB research, allowing detection of several confounding or risk factors contributing to TB treatment failure and to follow trends of drug resistant TB.

Tuberculosis as a social disease

Two thirds of new TB cases diagnosed in year 2000 were men and approximately 60% were economically unfortified or uninsured (TB register), in agreement with similar data obtained in New York and London in the 1990s. The highest prevalence of the TB was registered in areas of lower socioeconomic conditions and large immigrant populations (Hayward and Coker, 2000). At least 5% of TB patients in London and up to 25% in New York were homeless or had a history of residence in a hostel for the homeless (Hayward and Coker, 2000).

In south Estonia the incidence and risk factors of TB has been intensively studied in the city and county of Tartu in two periods, from 1985 to 1989 (Sillastu and Altraja, 1991) and from 1995 to 1999 (Lang *et al.*, 2001). The authors in both studies revealed that approximately 2/3 of patients were male and the majority of TB cases occurred in the most productive age groups, such as 25-54 years. The results also demonstrated that alcohol abuse was prevalent among subjects from both studies. Considering social groups, the latest study demonstrated that the unemployed and industrial workers were the most represented groups (Lang *et al.*, 2001).

Molecular epidemiology of *M. tuberculosis*

Analysis of the *M. tuberculosis* population structure by IS*6110*- associated RFLP may provide information about the evolutionary history and the dissemination of particular clones in a given geographic region (van Soolingen *et al.*, 1995). In addition, Kremer *et al.* (1999) have shown that different genetic markers used in strain typing for epidemiological analysis of TB may lead to the recognition of well-defined genotype families within the *M. tuberculosis* complex.

An extreme example of clonality of *M. tuberculosis* is the Beijing area where more than 85% of the isolates exhibited more than 66% similarity amongst their IS*6110* RFLP patterns. Given that the highest density of this genotype of *M. tuberculosis* was found in the Beijing area, this family of strains was designated the "Beijing" genotype. These strains apparently have had some selective advantage in the Beijing area and therefore, this family is an aggressively expanding clone that is spreading to neighbouring countries and other continents (van Soolingen *et al.*, 1995). However, more data are required, particularly on the relative fitness of this family of strains.

TUBERCULOSIS CHEMOTHERAPY AND DRUG RESISTANCE

One of the most meaningful advances in the history of TB has been the development of curative chemotherapy. Over the two centuries preceding this watershed, TB had taken roughly one billion human lives (Iseman, 2000). TB chemotherapy has three identifiable goals: to kill rapidly the massive numbers of bacilli that are multiplying in the tissues of the typically diseased host; to prevent the emergence of clinically significant strains of drug-resistant mutants and to sterilize effectively the site of disease.

Main principles of chemotherapy

As a result of more than 30 years of research in the treatment of TB (Bobrowitz and Robins, 1967; Fox and Mitchison, 1975; Snider *et al.*, 1984), the importance of combined chemotherapy has been clearly demonstrated. Medical therapy for TB requires the use of combinations of agents to ensure that clinically apparent infection is never treated with a single drug and also drug ingestion must continue uninterrupted for an adequate length of time to eradicate the infection (American Thoracic Society, 1986).

The goals of therapy can be described simply as being the provision of the most effective therapy in the shortest period of time with the most effective utilization of available resources. The major concepts involved in TB therapy in individuals with non-drug-resistant infection are: 1) all regimens should contain isoniazid (INH) due to effectiveness, low cost and tolerance; 2) treatment for less than 6 months leads to unacceptably high relapse rates; 3) pyrazinamide (PZA) should be used with isoniazid and rifampicin (RIF) for the first 2 months of treatment; 4) intermittent therapy is as effective as daily therapy if adequately supervised (Fox and Mitchison, 1975; Snider *et al.*, 1984).

Non-completion of TB treatment plays a key role in the prevalence and resistance patterns of this infection. Supervised or directly observed therapy (DOT), in which medication is taken at a facility under observation or a care provider brings the medications and observes that they are taken, is the most effective method for improving adherence to treatment (Sbarbaro and Johnson, 1968; McDonald *et al.*, 1982).

Regimens of 6 month's duration comprising an initial 2- month intensive phase of INH, RIF, PZA, with or without streptomycin (SM) or ethambutol (EMB), followed by INH and RIF can be expected to yield long-term success rates of 95% or greater for patients with drug-susceptible pulmonary disease (Iseman, 2000).

Mechanisms of development of drug resistance

David and Newman (David and Newman, 1971) reported in 1971 on the frequency of mutation yielding INH resistance for a standard laboratory strain of *M. tuberculosis*, H37Rv.
The authors considered various scenarios that might be associated with the evolution of drug-resistant strains. They calculated the time required for a strain to shift spontaneously (random genetic drift) from an INH-susceptible phenotype to a population with 1% resistance and compared this to the time observed to cause this drift by indirect selection (a complex system of enrichment with resistant mutants) and direct selective pressure (growth in the presence of INH). They found, according to this analysis, that the spontaneous shift would take 5,000 to 10,000 years; by contrast, 1% resistance evolved in only 200 days by indirect enrichment methods and as few as 5 to 6 days via the direct selection system. The average mutation rate bacterium/generation was 2.56×10^{-8}, giving rise to the calculated 5,000 to 10,000 years for spontaneous drift to 1% prevalence of INH-resistant mutants. In the direct selection method, cultures were exposed to either 0.2 or 1.0 µg/mL concentrations of INH, and mutations confirming resistance were noted to occur at 1.8×10^{-8} and $0.86 \times$

10^{-8} replications, respectively. Overall, the data from this report and previous analysis (David, 1970) indicates that the average mutation rate is 1.9×10^{-8} per bacterium per generation. Based on these observations, the authors concluded that the initial appearance of substantial populations of INH-resistant bacilli (more than 1.2×10^{-5} of the population) in a patient cannot be ascribed to spontaneous mutation but must reflect either prior exposure to INH or infection of that patient with a strain that has been preselected by treatment in another person. Thus, clinically significant levels of INH resistance are virtually always a human-generated phenomenon.

David (1970) earlier reported on the mutation rates (per bacterium, per generation) and average mutation frequencies in an unselected population of bacteria, for several standard anti-tuberculosis drugs. The mutation rates and prevalence of mutants, respectively, included the following: for INH (0.2 µg/mL) = 1.84×10^{-8} and 3.5×10^{-6}; RIF (1.0 µg/mL) = 2.2×10^{-10} and 1.2×10^{-8}; SM (2.0 µg/mL) = 2.9×10^{-8} and 3.8×10^{-6}; EMB (5.0 µg/mL) = 1.0×10^{-7} and 3.1×10^{-5}.

Although clinically significant resistance appears to evolve initially through the selective pressure of chemotherapy, there is no direct evidence to demonstrate how the interruption of therapy can contribute to the generation of resistance in *M. tuberculosis*.

Molecular genetic basis of drug resistance in *M. tuberculosis*

In order to develop improved strategies for diagnosis and therapy control of drug-resistant tuberculosis, a great effort has been invested toward defining the genetic basis of drug resistance (Musser, 1995; Cole and Telenti, 1995; Blanchard, 1996; Rattan *et al.*, 1998).

According to Musser and Sreevatsan, the remarkably restricted variation in *M. tuberculosis* complex structural genes has important implication for studies of drug resistance (Sreevatsan *et al.*, 1997). The lack of allelic diversity means that when amino acid polymorphism, or regulatory region nucleotide variation are observed, there should be strong suspicion that the variation has functional consequences, such as antibiotic resistance (Musser, 1995). The mutations in *M. tuberculosis* strains have been found to confer resistance only to a single drug or closely related members of a class. Findings of different studies are indicating that similar loci and mechanisms were involved whether there was resistance to one or more drugs within the individual strains (Heym *et al.*, 1994; Morris *et al*, 1995). These findings support the theory that resistance evolves by unlinked process, being acquired independently for one drug at a time.

The suggested mutation sites for the anti-tuberculosis activity of first-line drugs are *kat*G, *inh*A and *ahp*C for isoniazid, *rpo*B for the rifampicin, *rps*L or *rrs* for streptomycin, *emb*AB for ethambutol and *pcr*A for pyrazinamide (Iseman, 2000) (table 1).

Mutations associated with isoniazid resistance are located on several widely separated regions of the *M. tuberculosis* genome. Between 12 and 75% of isoniazid-resistant strains have been found to contain mutations either in codon 315 of the *kat*G gene or the *inh*A ribosomal binding site, and an addition 13 to 18% have contained mutations in the *ahp*C-*oxy*R intergenic region, often in conjunction with katG mutations outside

of codon 315. In contrast rifampicin resistance in 96% of the organisms is attributable to substitutions in a 27-amino-acid region of the beta subunit of RNA polymerase encoded by *rpo*B (Musser, 1995).

However, Victor *et al.* questions the assumption that all mutations confer resistance in genes in which mutations have been associated with antituberculosis drug resistance and highlights the importance of establishing the causal relationship between any given mutation and drug resistance (Victor *et al.,* 2001).

The most promising use of genotypic analysis is in the detection of the most worrisome MDR strains of *M. tuberculosis*. More than 96% of these should be identified through their rpoB mutations. However, for further guidance with therapy of these MDR strains, the use of genotypic methods will require more detailed understanding of resistance to second-line drugs: capreomycin, cycloserine, ethionamide, kanamycin, amikacin, PAS; as well as the low-level resistance mechanisms. Whether failure to detect the low-level resistant isolates by genetic methods has adverse clinical consequences remains to be determined.

Table 1. Gene loci involved in conferring drug-resistance in *Mycobacterium tuberculosis*.

Drug	Gene	Type and location of mutations	Reported freq. in resistant strains[a]	Reference
Rifampicin	***rpoB***	codon 526 &531* common (70%); codon 533, 510, 515; 508-509 may be polymorphisms	90-98%	*Telenti A., 1993; Ohno H., 1997; Rossau R., 1997; Telenti. 1997; Heym B., 1994; Morris S., 1997; Nachamkin I., 1997;Williams DL., 1994; Piatek AS.,1998*
Isoniazid	***katG***	S315T most common, K463L polymorphism in 15%-30%	22-64%	*Telenti A., 1997; Heym B., 1994; Morris S., 1997; Nachamkin I., 1997; Rouse DA., 1995; Piatek AS., 2000*
	inhA	Usually promoter mutation, low-level resistance	20-34%	*Telenti A., 1997;Heym B., 1994; Piatek AS., 2000*
	ahpC	Usually together with *katG* mutations	10%	*Telenti A., 1997; Deretic V.,1995;Sreevatsan S., 1997;*
	kasA	50% also with katG mutations; codon G269S found in wild type	14%	*Piatek AS., 2000*
Streptomycin	***rpsL***	Lys-43 (AAG) to Arg (AGG)	30-70%	*Dobner P., 1997; Heym B. 1997*
	rrs	491-C to T; 516-C to T; 905-A to G; 906-A to T	2-4% 2%	*Dobner P., 1997; Finken., 1993; Ramaswamy S., 1998; Meier A.. 1996;*
Amikacin/ Kanamycin	***rrs***	A1400G	67%	*Alangaden GJ., 1998; Suzuki Y., 1998;*
Fluoroquinolone	***gyrA***	Codon 95; polymorphism in 15% strains (Sreevatsan et al)	> 90%	*Xu C., 1996;*
Pyrazinamide	***pncA***	>26 different nucleotide substitutions, insertions, or deletions	72-96%	*Scorpio A., 1997; Sreevatsan S., 1997;*
Ethambutol	***emb*CAB**	Codon 36 in 90%	48-62%	*Telenti A., 1997; Sreevatsan S., 1997;*

[a] mutation frequencies are as determined by DNA sequencing technique and polymerase chain reaction-single strand conformational polymorphism analysis;

* associated with high levels of phenotypic resistance to RIF (>100 µg/mL)

TRANSMISSION OF TUBERCULOSIS WITHIN INSTITUTIONS

Typically TB is transmitted by aerial dissemination of small particles, which are inhaled by individuals sharing a closed environment with persons expressing active disease. Large outbreaks with high rates of infection and extensive disease have occurred in institutional or congregational settings, including hospitals (Pearson *et al.*, 1992), clinics (Fischl *et al.*, 1992; Beck-Sagué *et al.*, 1992), residential facilities for persons with AIDS (Daley *et al.*, 1992), nursing homes (Stead and Dutt, 1991; CDC, 1983), homeless shelters, and prisons (Coninx *et al.*, 1998; Portaels *et al.*, 1999; Pfyffer *et al* 2001; Drobniewski *et al.*, 2002). In these facilities TB has been transmitted to the other patients or clients as well as to health care professionals, lay staff, and visitors (Iseman, 2000).

Due to of their waxy coats, which limit fluid loss across the cell membrane of *M. tuberculosis*, they remain viable much longer than most other bacteria in an airborne or exposed state (Iseman, 2000). In addition, the small size of the bacteria (1 to 4 μm in length and 0.3 to 0.6 μm in diameter) may also prolong the circulation time of aerosols.

Nosocomial transmission of tuberculosis

Outside the U.S., recent studies of institutional transmission and nosocomial TB among patients and HCW have been reported from Italy (Di Perri *et al.*, 1989; Di Perri *et al.*, 1992) and France (Bader, 1992; Longuet *et al.*, 1995) and Russia (Narvskaya *et al.*, 2002). A recent report from Buenos Aires described an institutionally spread outbreak of MDR-TB. In a large referral hospital that served both complicated HIV and TB cases, 101 patients with MDR-TB were seen over 18 months from 1994 to 1995; 68 cases were proven to reflect on-site exposure (Ritacco *et al.*, 1997).

HCW have traditionally been considered at risk of acquiring TB due to unintentional exposures to undiagnosed disease in the work place (Barrett-Connor, 1979; Menzies *et al.*, 1995). The risk of TB amongst HCW was substantial in the era prior to the introduction of antibiotics (Sepkowitz *et al.*, 1995) but declined rapidly after 1950 due to a lower incidence of the disease in the population and the advent of effective therapy (Menzies *et al.*, 1995). However, attention has been refocused on these phenomena due to the rising prevalence of MDR-TB and high rates of morbidity and mortality among immunocompromised persons, particularly those with AIDS (Iseman, 2000). Before our study, the problem of nosocomial transmission of TB among patients and HCW has not been addressed in Estonia.

AIMS OF THE STUDY

Tuberculosis infection and disease patterns among different populations are extremely heterogeneous. The overall aim of present investigation was to assess mainly by microbiological methods the epidemiology of tuberculosis in Estonia. Furthermore, the different features of MDR *M. tuberculosis* were studied.

The specific objectives were to:

- investigate the prevalence of drug resistant TB in Estonia: countrywide study in 1994,

- assess the role of clonality of *M. tuberculosis* involved in the spread of drug resistant TB by using two molecular methods (IS*6110*-RFLP and spoligotyping),

- study the incidence of tuberculosis among health care workers;

- investigate the means by which drug resistance evolves among drug-susceptible *M. tuberculosis* strains during antiTB treatment,

- determine drug susceptibility of MDR *M. tuberculosis* clinical isolates to second-line and alternative drugs,

- study 16S rRNA mutations in kanamycin-resistant but amikacin-susceptible *M. tuberculosis* clinical isolates.

Materials and Methods

Subjects and microbial strains (Paper I – VI)
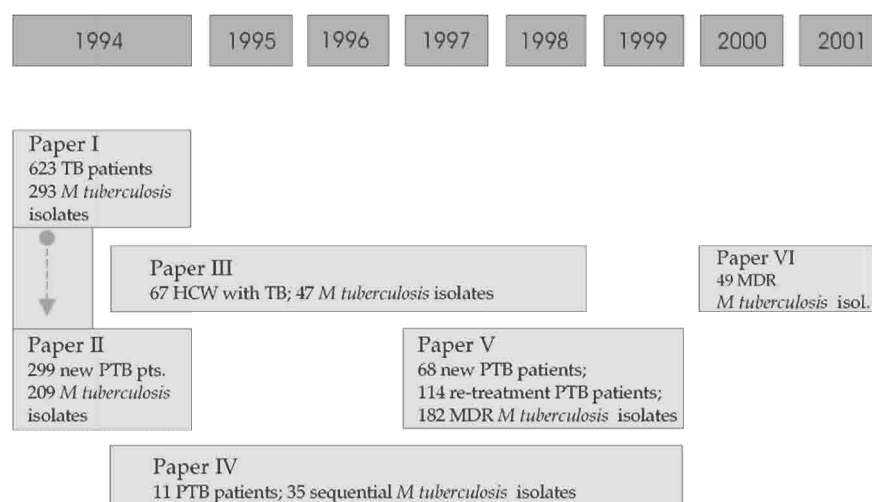

Figure 3. The study populations and time periods.

Paper I. In 1994 during a countrywide study, 623 TB cases were reported in Estonia. From these 623 cases, 518 were reported as new cases, i.e. with no previous history of TB (355 men and 163 women, mean age 41 years), and 105 as relapses (81 men and 24 women, mean age 56 years). Of the 518 new cases, 302 (58.3%) were culture positive for *M. tuberculosis*. Of the 105 cases with a history of previously treated TB (relapses), 27 (25.7%) had culture-verified TB. In total 293 *M. tuberculosis* isolates with 266 isolates from newly diagnosed pulmonary TB patients and 27 isolates from relapse cases were available for analysis.

Paper II. Altogether 209 (70%) isolates were obtained from 299 newly diagnosed smear and/or culture confirmed pulmonary TB cases all over Estonia and further analyzed with molecular techniques. Three cases among newly diagnosed pulmonary TB patients in 1994 were later reclassified as previously treated cases.

Paper III. The HCW eligible for the study were full-time employees registered in the databases of different health care institutions in Estonia. A total of 67 HIV-negative HCW among the 14 730 in the HCW registers were diagnosed as patients with primary TB during calendar years 1994 -1998. Altogether, 47 *M. tuberculosis* isolates were available for drug susceptibility testing.

Paper IV. During a six-year period (1994-1999), 35 sequential isolates of *M. tuberculosis* from 11 (78.6% out of all possible candidates) TB patients initially diagnosed as drug susceptible pulmonary TB cases and having at least three isolates tested for drug susceptibility with the interval between isolates ≥2 months, were analyzed from the Estonian National Reference Laboratory (ENRL) archive and

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included to the study. Altogether, 11 were divided into two different patient groups. Group 1: 6 cases characterized by continuous excretion of drug susceptible *M. tuberculosis* isolates for more than 10 months, despite anti-tuberculosis treatment. Group 2: 5 cases characterized by continuous excretion of *M. tuberculosis* isolates of different drug susceptibility patterns.

Paper V. During a three-year period (1997-1999) 182 clinical isolates of MDR *M. tuberculosis* were isolated from 68 new and 114 re-treatment pulmonary TB patients who were considered for chemotherapy with second-line and alternative drugs.

Paper VI. Forty kanamycin-resistant but amikacin-susceptible, five kanamycin- and amikacin-susceptible and four kanamycin- and amikacin-resistant *M. tuberculosis* clinical isolates from the archive of the ENRL were selected for the study.

Collection and storage of strains

Clinical samples of patients (Paper I and II) were collected by the hospital personnel or TB doctors and sent to the TB Reference Laboratory in Tartu (catering for South Estonia, approximately 500 000 population) or to TB laboratory in Kivimäe Hospital (catering for a population of approximately 1 million) as routine samples for microbiological investigation. All available isolates obtained in both laboratories were further shipped to SMI (Smittskyddsinstitutet; International Reference laboratory for Estonia), in Stockholm for retesting of drug susceptibility. The bacterial isolates were stored in Middlebrook-Glycerin media at -70°C and were also used as the material for paper II.
Sputum samples from TB patients (Paper IV) in the follow up period of TB treatment efficiency monitoring were collected by TB doctors as routine samples for microbiological examination. The *M. tuberculosis* isolates were stored in skim milk at -70°C.
Also samples from HCW (Paper III) were collected and handled as routine samples by different TB laboratories in Estonia.
In paper V 182 clinical isolates of MDR *M. tuberculosis* were isolated from 68 new and 114 re-treatment TB patients who were considered for chemotherapy with second-line drugs. All samples were submitted to the reference laboratory as routine samples for microbiological examinations and the isolates were stored in skim milk at - 70°C.
For paper VI 49 clinical isolates of *M. tuberculosis* was selected from the ENRL TB strain collection/archive and the isolates were kept as in earlier studies.

Primary isolation of *M. tuberculosis*

In countrywide study *M. tuberculosis* complex isolates were isolated in the Mycobacterial Laboratory in Tallinn (central laboratory for the North-Estonia) and at the ENRL in Tartu. Culture was performed on L-J medium. The specimens were inoculated in one tube containing L-J medium and in one containing L-J medium with 0.6% pyruvate. The tubes were then incubated at 37°C for seven weeks and examined weekly for mycobacterial growth.

Identification of mycobacterial strains

The species identification of the isolates was based on standard microbiological tests: colony morphology, acid fast staining, biochemical tests (Isenberg, 1992) and was confirmed with a DNA-RNA hybridization technique (AccuProbe; GenProbe Inc., San Diego, Calif.) at the SMI in Stockholm or in the ENRL in Tartu.

Drug susceptibility testing

In Paper I and II drug susceptibility testing of all isolates was performed both in Estonia and in Sweden. In Estonia it was carried out by conventional culture on solid medium using the proportion method (Canetti *et al*, 1969), and at SMI it was done using radiometric respirometry according to the Bactec system (Becton Dickinson, Sparks, MD) (Siddiqi, 1995). This method is in good agreement with the resistance ratio method on L-J medium (Hoffner and Källenius, 1988). The drugs tested included streptomycin (4 µg/ml), isoniazid (0.2 µg/ml), ethambutol (5 µg/ml) and rifampicin (2 µg/ml).

Isolates obtained from HCW (Paper III) were tested prior to 1995 in different TB laboratories in Estonia on solid media using proportion method. Since 1995 all isolates were tested only either in Tartu or Tallinn with the proportion method on the solid medium or by the Bactec system.

In Paper IV all isolates were tested at the ENRL in Tartu by the Bactec 460 radiometric system.

Susceptibility testing of second-line and alternative drugs: amikacin (4 µg/ml), azithromycin (4 µg/ml), capreomycin (10 µg/ml), ciprofloxacin (2 µg/ml), clarithromycin (2 µg/ml), clofazimine (2 µg/ml), cycloserine (50 µg/ml), ethionamide (5 µg/ml), p-aminosalicylic acid (8 µg/ml), thiacetazone (2 µg/ml) were determined using the Bactec 460 radiometric system with Middlebrook 7H12 broth and the same standard protocol was used as for the susceptibility testing of first-line drugs (Siddiqi, 1995).

Susceptibility testing of second-line/alternative drugs was done at SMI, Stockholm, Sweden (Paper V).

Determination of Minimal Inhibitory Concentration (MIC)

MICs were determined in Middlebrook agar 7H10 supplemented with OADC as well as 2 - 256 µg/mL of kanamycin or amikacin. The lowest drug concentration that produced complete inhibition of the bacterial growth was determined as MIC. Resistance to AK and KM was defined as a MIC of >4 µg/mL (Pfyffer *et al.*, 1999; Heifets, 1991a). The MICs determination was done at SMI.

Standard DNA typing for *M. tuberculosis* complex isolates

RFLP analyses. Extraction of DNA from mycobacterial strains and DNA fingerprinting with IS*6110* as a probe were performed using standardized methods (van Embden *et al.*, 1993) at SMI. In brief, after 3 to 4 weeks of growth on L-J medium, the bacteria were harvested and heat killed (80°C for 20 min). DNA was extracted and digested with *Pvu*II. Following electrophoresis of the digested DNA on

an agarose gel, a 245-bp fragment of IS6110 was chemiluminescence-labeled. The gels were scanned and results were analyzed by computer using the Gelcompar software (Applied Maths, Kortrijk, Belgium) as previously described (Heersma et al., 1998).

Similarity matrices were generated to visualize the relatedness between the banding patterns of all isolates. Isolates with banding patterns whose similarity coefficients were ≥65% (sharing more than two-thirds of the IS-containing PvuII fragments) were defined as belonging to a family of strains (Kremer et al., 1999; van Embden et al., 1993). Clinical isolates with identical IS6110 RFLP pattern constituted one cluster.

Spoligotyping. The spoligotyping method, with a slightly lower level of discrimination than that of IS6110 RFLP typing (Alto et al., 1999, Kremer et al., 1999), was used as an additional tool for determining relationships among the isolates (van Soolingen et al., 1995). The DR (direct repeat) locus in the M. tuberculosis complex genome contains multiple highly conserved 36-bp direct repeats. The repeats are separated by 35- to 41-bp spacer sequences, which are variable. The analysis of this locus by spoligotyping is based on the unique nature of these separate spacer sequences and their ability to be hybridized to synthetic spacer-oligonucleotides that are bound to a membrane. The hybridisation pattern demonstrates which spacer oligonucleotides are present in each strain (Kamerbeek et al., 1997). The DR-based fingerprint is a good secondary marker to support or rule out strain clustering.

Purified chromosomal DNA from RFLP typing was also available for spoligotyping. Membranes for spoligotyping were obtained from Isogen, Bioscience BV, Utrecht, The Netherlands. The membranes contained oligonucleotides derived from the spacer DNA sequences, interspersed with the directly repeated sequences in the DR region of M. tuberculosis strain H37Rv and M. bovis BCG. The presence or absence of these spacers in M. tuberculosis complex strains can be detected by using hybridisation of the amplified DNA for these spacer regions by primers complementary to the DR. PCR and hybridisation was performed as previously described (Kamerbeek et al., 1997).

DNA Sequencing

Among the many techniques used to identify drug resistance-associated mutations, automated DNA sequencing of PCR products has been the most widely applied (Ramaswarmy et al., 1998). Automated DNA sequencing using fluorescent/infrared primer or terminator labeling and a variety of detection systems, coupled with software-based sequence determination, has greatly improved the ease, speed, accuracy, and reliability of DNA sequencing.

To analyze the area where kanamycin and amikacin resistance mutations have previously been reported (Alangaden et al., 1998;Ramaswamy et al., 1998; Suzuki et al., 1996), an approximate 350 bp region of the 16S rRNA gene was amplified and sequenced. Each 50μl PCR reaction contained 10 ng of genomic DNA, 3mM $MgCl_2$, 400 μM of each dNTP, 10 μl 10* PCR buffer, 2U of AmpliTaq gold (Applied Biosystems, NJ, US), 20 pmol of each primer rrs.PCR.F123 (AAGGGCTGCGATGCCGCGAG) and rrs.PCR.R535 (AAGTCCGAGTGTTGCCTCAGG) respectively. The reaction was initiated at 95° C for 10 minutes, followed by 30 cycles of (95° C 30 sec, 56° C 30 sec and 72°C 30 sec) and finally elongated for 7 min at 72° C. PCR products were subjected to a

purification step by GFX™ PCR Purification Kit (Amersham Pharmacia Biotech). Each sample was analyzed in both the forward and reverse direction using Big Dye DNA sequencing kit (Applied Biosystems, Warrington UK), the rrs 123 and the rrs 535 primers respectively. The reaction mixtures were precipitated according to the Applied Biosystems protocol for purifying extension products and analyzed in an ABI prism 3100 genetic analyzer.

Ten MDR *M. tuberculosis* isolates having different resistance to KM, AK and SM were selected for additional sequencing of the complete rrs gene. All sequences obtained were compared and substitution positions were numbered according to the CDC 1551 public rrs gene (Accession number- AE 007009).

Statistical methods

Data analysis was performed using the Statistica and Sigmastat programs. Subject variables were examined using the χ^2 test of association for categorical variables and t test for continuous variables. $P < 0.05$ was used to indicate statistical significance.

RESULTS

Drug susceptibility patterns of *M. tuberculosis* isolates

Paper I. In total 266 of 302 pulmonary isolates derived from new TB cases, 91 from Tartu and 175 from Tallinn, were tested for drug susceptibility. Of the 266 isolates 75 (28.2%) were resistant to one or more of the drugs tested (table 2), and 27 (10%) were resistant to both isoniazid and rifampicin. Thus, 10% isolates were multi-drug resistant, i.e. resistant to at least isoniazid and rifampicin.

Table 2. Drug resistant *M. tuberculosis* strains in 266 new TB patients in Estonia 1994

Drug	Number of resistant strains (%)
Isoniazid	58 (21.8)
Rifampicin	27 (10.0)
Streptomycin	56 (21.0)
Ethambutol	19 (7.1)
One or more drugs	75 (28.2)
Isoniazid + Rifampicin	27 (10.0)
All four drugs	12 (4.5)

There were 27 culture-positive patients with a history of previously treated TB. Of these isolates from these patients 13 (48%) were isoniazid resistant, 6 (22%) rifampicin resistant, 11 (41%) streptomycin resistant and 6 (22%), were resistant to ethambutol, 15% of the strains were multidrug resistant. Among relapse cases drug resistance to isoniazid, rifampicin and streptomycin were almost twice and to ethambutol thrice as frequent than among newly diagnosed pulmonary TB cases.

The *M. tuberculosis* isolates obtained from newly diagnosed pulmonary TB patients within the frame of this study were the basis for the subsequent molecular epidemiology study.

Paper III. From 47 *M. tuberculosis* isolates available for drug susceptibility testing, 23 (49%) were resistant to at least one of the drugs, including streptomycin, isoniazid, rifampicin, or ethambutol. Eighteen (38.3%) isolates were multidrug-resistant. The combinations of drug resistances are shown in Table 3.

Table 3. Patterns of anti-tuberculosis drug resistance of 47 *M. tuberculosis* strains isolated from HCW in comparison with initial resistance rates in 1994[1] and 1998[1]

	Strains of HCW tested		Total in Estonia in 1994[1]		Total in Estonia in 1998[1]	
	n.	%	n.	%	n.	%
Total	47	100	266	100	376	100
Fully susceptible	24	51	191	72	237	63
Mono-resistance	1	2	30	11	51	14
Isoniazid (INH)	*0*	*-*	*11*	*41*	*10*	*3*
Rifampicin (RIF)	*1*	*2*	*0*	*-*	*1*	*<1*
Ethambutol (EMB)	*0*	*-*	*2*	*1*	*1*	*<1*
Streptomycin (SM)	*0*	*-*	*17*	*6*	*39*	*10*
MDR	*18*	*38*	*27*	*10*	*53*	*14*
INH+RIF	*1*	*2*	*6*	*2*	*1*	*<1*
INH+RIF+EMB	*0*	*-*	*0*	*-*	*2*	*<1*
INH+RIF+SM	*4*	*9*	*9*	*3*	*18*	*5*
INH+RIF+SM+EMB	*13*	*28*	*12*	*5*	*32*	*9*
Other resistance	*4*	*9*	*18*	*7*	*35*	*9*
INH+SM	*2*	*4*	*13*	*5*	*28*	*7*
INH+EMB+SM	*1*	*2*	*5*	*2*	*5*	*1*
RIF+SM	*1*	*2*	*0*	*-*	*0*	*-*

[1] Only new cases of TB included.

The analysis of drug susceptibility patterns of *M. tuberculosis* strains obtained from HCW demonstrates that half of the culture-confirmed cases were infected with bacterial strains having resistance to at least one anti-tuberculosis drug tested. The number of MDR *M. tuberculosis* isolates detected among HCW was three times higher than among the general population during the same time period. Also, among the HCW the high numbers of *M. tuberculosis* strains (28%) were resistant to all four anti-tuberculosis drugs tested.

In 15 (22%) cases, both smear and culture results were negative. In four cases culture results were not obtained, and in one case, TB was confirmed only by histo-pathological examinations.

Paper V. One hundred eighty two MDR *M. tuberculosis* isolates were tested with the radiometric Bactec 460 system to first-, second-line and alternative drugs.
The final DST results of 182 MDR *M. tuberculosis* clinical isolates, originated from newly diagnosed and re-treatment MDR-TB cases in 1997 to 1999 are given in Table 4.

Table 4. Antimicrobial susceptibility of MDR *M. tuberculosis* clinical isolates, obtained from newly diagnosed and re-treatment TB cases in 1997 to 1999.

Drug	Drug conc. used (μg/mL)	Total no of isolates tested	No of resistant isolates (%)	
Primary drugs[‡]				
Isoniazid	0.2	182	182	(100)
Rifampicin	2.0	182	182	(100)
Streptomycin	4.0	182	168	(92)
Ethambutol	7.5	182	138	(76)
Second-line and alternative drugs				
Ethionamide	5.0	182	48	(26)
Amikacin	4.0	182	44	(24)
Ciprofloxacin	2.0	168	15	(9)
Clarithromycin	2.0	165	14	(9)
Capreomycin	10.0	182	5	(3)
Clofazimine	2.0	158	0	(0)
Azitromycin[§]	4.0	126	126	(100)
p-aminosalicylic acid (PAS)[§]	8.0	170	160	(94)
Thiacetazone[§]	2.0	170	151	(89)
Cycloserine[§]	50.0	170	139	(82)

[‡] Drug susceptibility testing was done by radiometric Bactec system at the Estonian National Reference Laboratory in Tartu on routine bases.

[§] Drug susceptibility testing results to these drugs were considered unreliable.

Due to methodological problems in testing cycloserine, thiacetazone, p-aminosalicylic acid, and azithromycin with Bactec system, the results of these drugs were omitted from further analyses.

Very similar drug susceptibility to primary, second-line and alternative drugs in both newly diagnosed and re-treatment cases were found throughout the study period (figure 4).

Over the three years of the study period, the resistance among the newly diagnosed MDR-TB patients was stable for most drugs (figure 5). To ciprofloxacin and ethionamide, however, more resistant cases were detected in 1999 than in previous years.

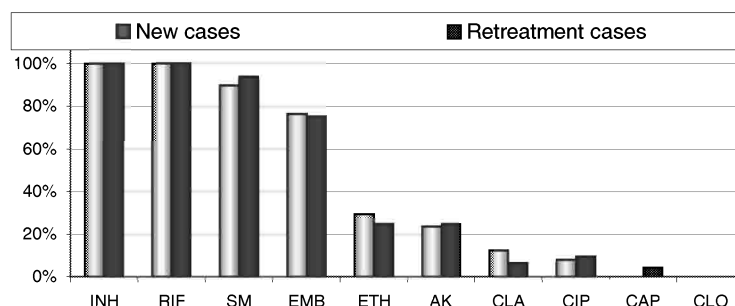**Figure 4.** Drug resistance of MDR *M. tuberculosis* to different drugs.

Figure 5. The trends of drug resistance among the newly diagnosed MDR-TB patients.
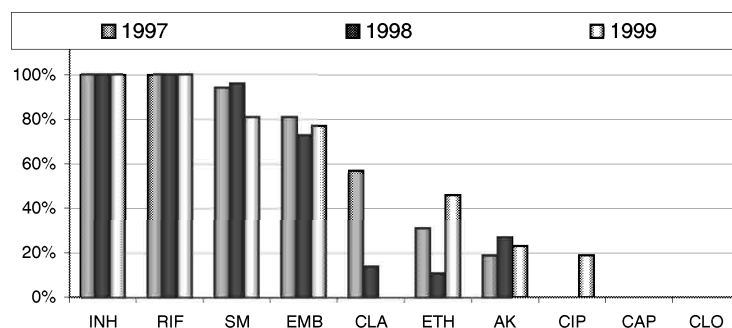
Minimal Inhibitory Concentrations (MIC)

Paper VI. Forty isolates had MICs of KM from 8 to 32 µg/mL and were susceptible to AK (MIC ≤1µg/mL). In one isolate the MIC of KM was 64 µg/mL and the MIC of AK was 8 µg/mL. Three isolates were included as dual resistant controls in the assay and had MICs >256 µg/mL to both drugs while five isolates were included as susceptible controls had MIC ≤1 µg/mL to AK and MIC 1-4 µg/mL to KM.

Molecular typing of *M. tuberculosis* isolates

IS*6110* RFLP typing

Paper II. In the study 209 *M. tuberculosis* isolates were characterized by IS*6110* RFLP analysis. The number of IS*6110* copies per isolate varied between 5 and 19 (mean of 12 bands). The majority, i.e. 195 (93%) of the isolates, contained 8 to 17 copies, with a mean of 12 bands.

M. tuberculosis isolates from 102 of the 209 patients (49%) belonged to different clusters, while 107 (51%) clinical isolates presented unique (individual) RFLP fingerprint patterns.

A total of 140 isolates (67%) (44 with unique patterns and 96 with clustered ones) could be allocated into five major families on the basis their RFLP patterns (≥65% similarity), families A to E (figure 6). The largest family, family A, comprised 61 isolates in 12 clusters, with largest cluster comprising 10 closely related or identical isolates (table 5). Of the 209 patients 47 (22%) were infected with isolates belonging to families B to E, and 34 (16%) isolates belonged to 12 clusters outside the five major families (figure 6; table 5).
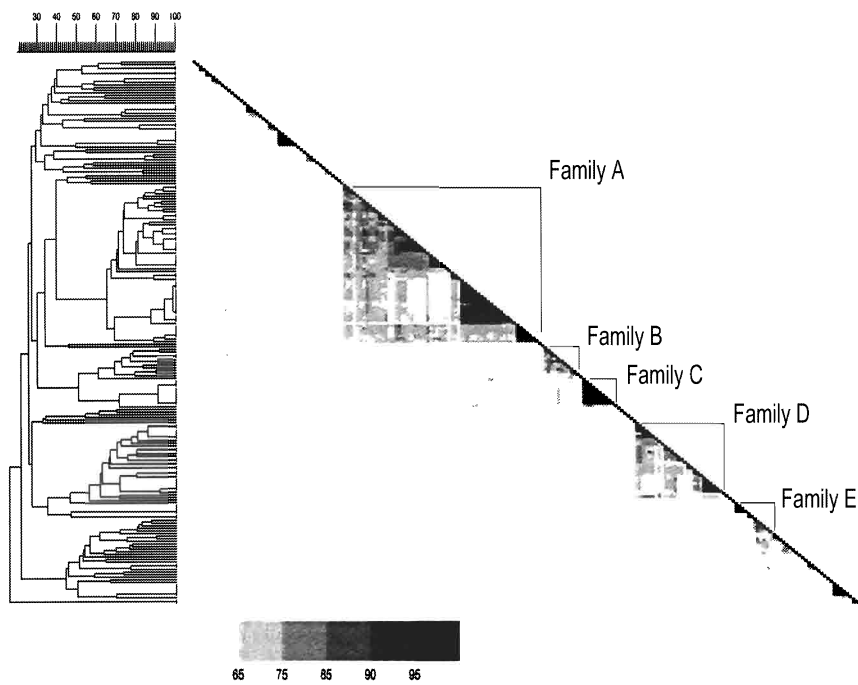
Figure 6. Similarity matrix showing the relatedness between the RFLP banding patterns of 209 *M. tuberculosis* isolates. The different RFLP patterns divided the isolates into five major families (family A; B; C; D; and E). The value of the similarity coefficient between values of 65 and 100% is depicted by the five different grey tones in the matrices. The diagonal is formed by the 100% similarity coefficient values of the corresponding strains.
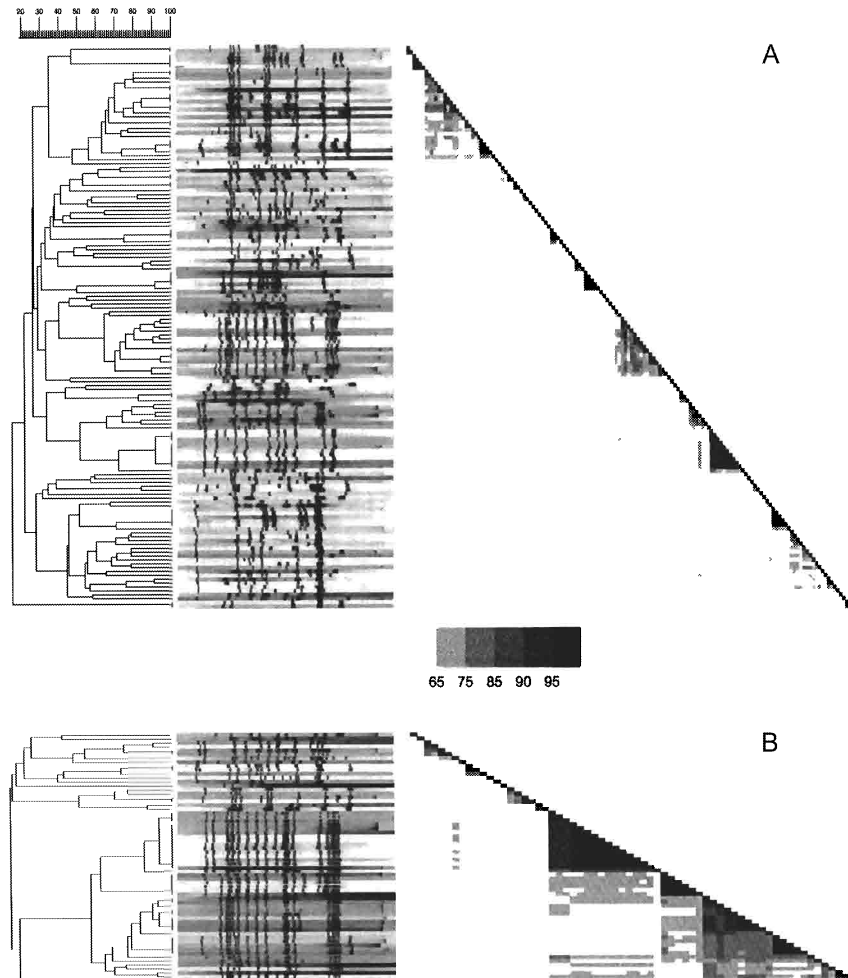
Figure 7. IS*6110* fingerprint patterns of the 145 drug susceptible (A) and 64 drug-resistant (B) isolates and the corresponding dendrogram and similarity matrixes. Banding patterns are ordered by similarity. The position of each IS*6110* band is normalized so that the banding patterns of all isolates are mutually comparable. Band positions were determined by using the peak finder function of the Gelcompar software and were controlled manually by comparison with the original IS*6110* autoradiogram. The fingerprint patterns were analysed for similarity by using the Dice coefficient, and a dendrogram was calculated with the unweighted pair group method using average linkage as specified by the supplier.

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Table 5. Drug resistance patterns of 140 *M. tuberculosis* isolates clustered with the families A to E

Family	Cluster	No of isolates	Drug resistance pattern[b]	Susc.	Any res.	MDR
A	A_1	2	AB I+ AB II	1	1	0
	A_2	2	AB I	2	0	0
	A_3	2	AB I	2	0	0
	A_4	2	AB II	0	2	0
	A_5	2	AB IV	0	2	2
	A_6	4	AB IV	0	4	4
	A_7	5	AB III + AB V	0	5	0
	A_8	2	AB I	2	0	0
	A_9	3	AB III+ AB IV	0	3	2
	A_{10}	10	AB I + AB III + AB IV + AB V	1	9	4
	A_{11}	3	AB IV + AB V	0	3	2
	A_{12}	4	AB II + AB IV	0	4	1
	A^a (SC 65-99%)	20	AB I + AB II + AB III + AB IV	10	10	6
	Total	61		18	43	21
B	B_1	2	AB II+ AB IV	0	2	1
	B^a (SC 69-92%)	9	AB I + AB II + AB III	6	3	0
	Total	11		6	5	1
C	C_1	3	AB I	3	0	0
	C_2	7	AB I	7	0	0
	Total	10		10	0	0
D	D_1	2	AB I+ AB II	1	1	0
	D_2	3	AB I	3	0	0
	D_3	2	AB I	2	0	0
	D_4	2	AB I	2	0	0
	D_5	2	AB I	2	0	0
	D^a (SC 66-86%)	6	AB I+ AB II	5	1	0
	Total	17		15	2	0
E	E_1	2	AB I+ AB IV	1	1	1
	E_2	5	AB I+ AB II	3	2	0
	E^a (SC 92%)	2	AB I + AB II	1	1	0
	Total	9		5	4	1
Other	F_1	2	AB I	2	0	0
	F_2	2	AB I+ AB III	1	1	0
	F_3	2	AB I+ AB II	1	1	0
	F_4	3	AB I	3	0	0
	F_5	2	AB I	2	0	0
	F_6	6	AB I+ AB II	5	1	0
	F_7	4	AB I	4	0	0
	F_8	2	AB I	2	0	0
	F_9	2	AB II	0	2	0
	F_{10}	5	AB I	5	0	0
	F_{11}	2	AB I	2	0	0
	Total	32		27	5	0

[a] Contains isolates which did not cluster but belonged to a family having a similarity coefficient of ≥65%.

[b] AB I, susceptible isolates; AB II, monoresistant isolates; AB III, isoniazid- plus streptomycin-resistant isolates; AB IV, MDR isolates; AB V, isoniazid- plus streptomycin- plus ethambutol-resistant isolates.

Paper IV. A total of 35 *M. tuberculosis* serial isolates from 11 patients were analyzed. The interval between the obtaining the samples for the first and the last isolate from each patient varied from 313 to 1201 days (mean, 828 days), for group 1, and from 370 to 1850 days (mean, 1091 days) for group 2 ($P = 0.3$) (figure 8). Within group 1, 5 patients had a follow-up isolate with an IS*6110* RFLP pattern that was identical to the pattern of the first isolate (figure 9). One patient had a second isolate with an IS*6110* RFLP pattern that slightly differed from that of the first isolate (1-band difference; loss of 1 band), whereas a third isolate from that patient was indistinguishable from the first. In group 2, 5 patients had follow-up isolates with IS*6110* RFLP patterns that clearly differed from the pattern of the first isolate, and, in 2 cases, the patterns of the first and second isolates differed from that of the third (figure 9). In all such cases, these changes have been considered, after the possibility

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for laboratory cross-contamination was ruled out, to reflect the appearance of a new *M. tuberculosis* strain.

Figure 8. Time intervals between sequential isolates of TB patients (*Group* 1 and *Group* 2)

Drug susceptible isolates of *M. tuberculosis*

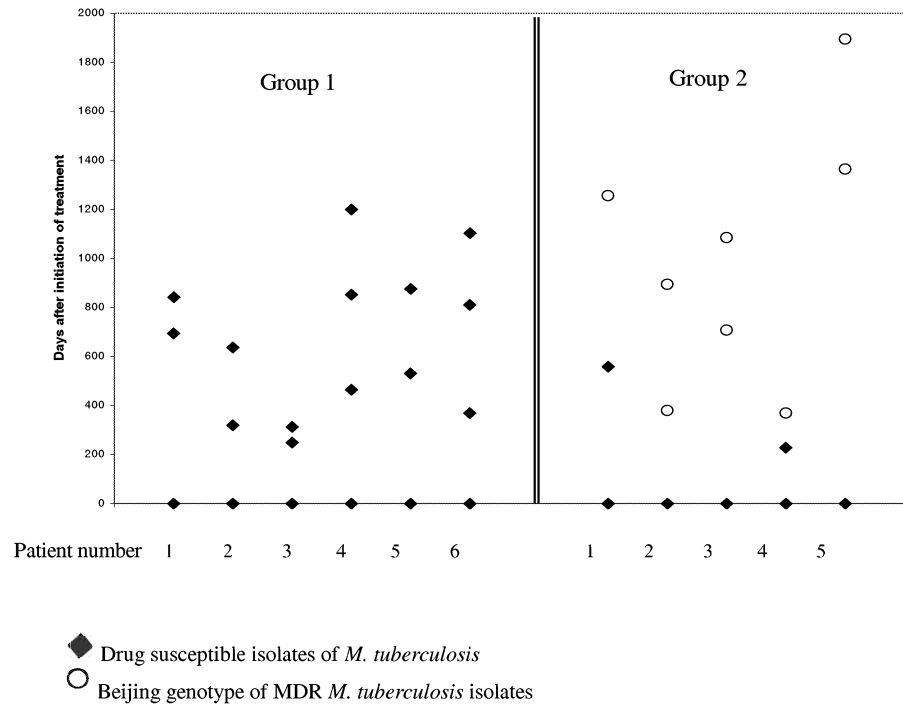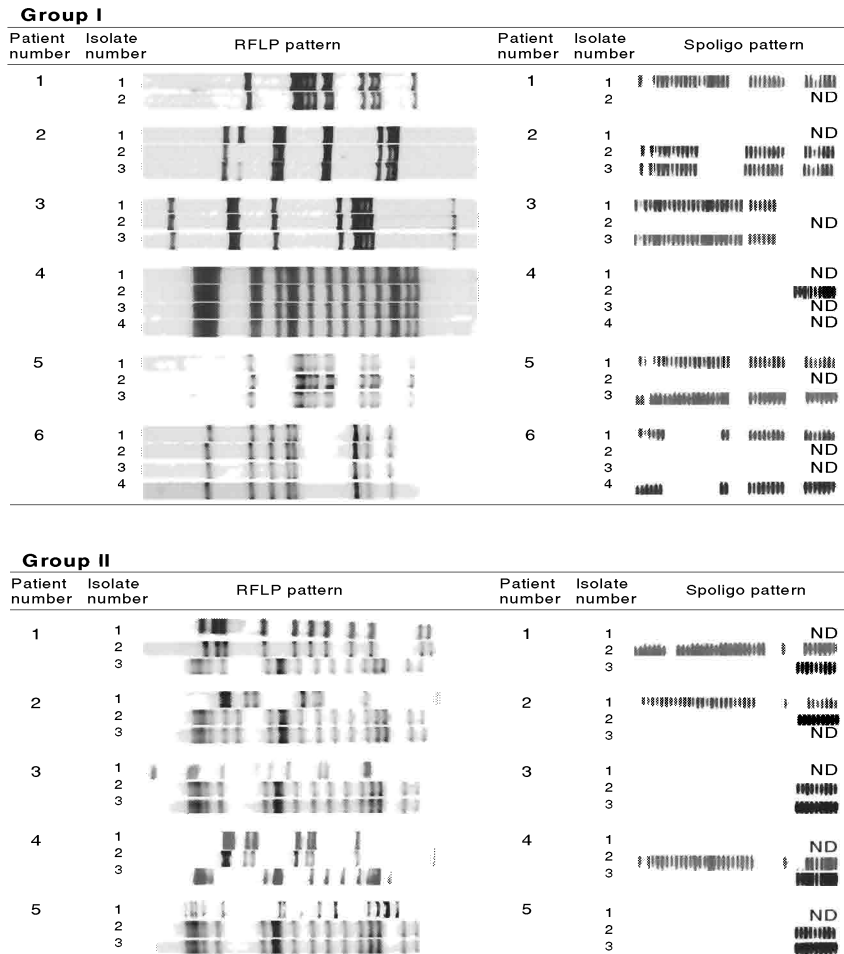Beijing genotype of MDR *M. tuberculosis* isolates

Figure 9. DNA fingerprinting of *M. tuberculosis* related to the patient group 1 and 2.

ND, not determined.

Paper VI. The 49 *M. tuberculosis* isolates yielded 13 fingerprint patterns forty were grouped into 4 fingerprint-defined clusters. Twelve of these were members of the *M. tuberculosis* Beijing genotype family (data not shown).

Spoligotyping

Paper II. Thirty-seven isolates of different RFLP pattern were selected for spoligotyping, according to the similar distribution of different families/clones/non-related strains in the investigated samples. The twelve strains from family A showed an identical and unusual spoligopattern, lacking spacers 1 to 34. This pattern is consistent with the spoligotype pattern of the so-called Beijing genotype *M. tuberculosis* strains (van Soolingen *et al.*, 1995). The spoligotypes of the non-Beijing family strains were highly diverse (figure 10).

Figure 10. Spoligopatterns of 37 *M. tuberculosis* isolates showing representative examples of families A to E and other clustered and nonclustered strains. The isolates are sorted in the same order in which they were sorted by their IS*6110* RFLP patterns in Figure 3. x, positive hybridisation signals; lack of hybridisation.

Isolate	Spoligotype	IS*6110* Cluster	No of IS*6110*
355/94	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx····xxxxxxx	F₁	11
3734/94	x·xxxxxxxxxxx·xxxxxxxxxxxxxxxxxxx····xxxxxxx	F	9
740/94	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx····xxxxxxx	N	17
704/94	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx····xxxxxxx	F₁	9
3775/94	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx····xxxxxxx	F	9
1155/94	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx····xxxxxxx	NC	10
2937/94	xxxxxxx·xxxxxxxxxxxxxxxxxxxxxxxxx····xxxxxxx	F	10
3748/94	xxxxxxxxxxx·xxxxxxxxxxxxxxxxxxx····xxxxxxx	NC	9
3505/94	xxxxxxxxxxxxxxxxxxxxxxxx·····x····xxxxxxx	NC	9
2661/94	xxxxxx·xxxxxxxxxxxxxxxxxxxxxxxx····xxxxxxx	NC	11
461/94	································xxxxxxxxxx	NC(FamA)	14
1832/94	································xxxxxxxxxx	A	15
1716/94	································xxxxxxxxx	A	15
2403/94	································xxxxxxxxxx	A	15
1277/94	································xxxxxxxxxx	A	17
1550/94	································xxxxxxxxxx	A	17
3032/94	································xxxxxxxxxx	A	17
2935/94	································xxxxxxxxxx	A	17
3942/94	································xxxxxxxxxx	NC(FamA)	17
904/94	································xxxxxxxxxx	A	16
1001/94	································xxxxxxxxxx	A	16
2356/94	································xxxxxxxxxx	NC(FamA)	18
1004/94	xxxxxxx··xxxxxxxxxxxxxxxxxxx···x····xxx·xxx	NC(FamB)	10
2011/94	xxxxxxx··xxxxxxxxxxxxxxxxxxx···x····xxxxxxx	NC(FamB)	12
3754/94	xxxxxxxxxxxxxxxxxxxxx····xxxxxxxxx····xxxxxxx	NC	13
205/94	xxxxxxx··xxxxxxxxxxxxxxxxxxx···x·····xxxxxxx	C	10
1221/94	xxxxxxxxxxxx·xxxxxxxxxxxxxxx···x····xxx·xxx	NC	6
631/94	xxxxxxxxxxxx·xxxxxxxxxxxxxxx···x·····xxxxxxx	NC	8
3674/94	xxxxxxxxxxx·xxxxxx····xxxxxxxx····xxxxxxx	D	10
1084/94	xxxxxxxxxxxxxxxxxx····xxxxxxxx····xxxxxxx	D	10
1485/94	xxxxxxxxxxxxx··········xxxxxxxx····xxxxxxx	D	9
696/94	xxxxxxxxxxxx··········xxxxxxxx····xxx·xxx	D₁	7
795/94	xxxxxxxxxxxxxxxxxx····xxxxxxxx····xxxxxxx	F	11
917/94	x··xxxxxxxxxxxxxxxx····xxxxxxxx····xxxxxxx	F	11
865/94	xxxxxxxxxxxxxxxxxxxxxxxxxx·x····xxxxxxx	NC	8
1667/94	xxxxxxxxxxxxxxxxx·xxxxxxxxxxx·x····xxxxxxx	NC	12
2900/94	xxxx·xxxxxxxxxxxxxxxxx······x····xxxxxxx	NC	11

Paper IV. Twenty *M. tuberculosis* isolates were characterized by spoligotyping. In group 1, the spoligotypes of isolates obtained from the same patient did not vary during the study period. In all patients in group 2, the spoligotypes of the first and second isolates differed. All MDR *M. tuberculosis* isolates identified in group 2 had an identical spoligotype, one that belongs to the Beijing genotype (van Soolingen *et al.*, 1995) (figure 9). The interval between the point at which the drug-susceptible *M. tuberculosis* isolates and the point at which MDR isolates were recovered from samples varied from 142 to 1364 days (mean, 617 days).

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DNA sequencing

Paper VI. *16S rRNA sequencing the 1400 region of the rrs gene.* All three highly KM-AK cross-resistant *M. tuberculosis* isolates revealed a guanine for adenine substitution at the 16S rRNA position 1400. The remaining 41 isolates, having MICs below or equal to 64 μg/mL of KM did not have any mutations in 1400 region of the *rrs* gene.

16S rRNA sequencing the whole rrs gene. We selected 10 *M. tuberculosis* isolates to examine possible mutations in the remaining approximately 70% of the nucleotide sequence of the gene. Two isolates, one having MIC 32 μg/mL and the other having the MIC 64 μg/mL to KM, had a thymine for cytosine substitution at the 16S rRNA position 516 (table 6). Five isolates having MICs between 1.0 to 16 μg/mL, and two isolates with the MIC 32 μg/mL to KM did not have mutations in the *rrs* gene.

Table 6. The sequencing results of whole *rrs* gene analysis in 10 *M. tuberculosis* isolates having different resistance profiles to KM, AK and SM.

Clinical isolates of *M. tuberculosis*[a]	Susceptibility to aminoglycosides[b]			Mutations in the rrs gene
	KM (MIC in μg/mL)	AK (MIC in μg/mL)	SM	
BTB 02-063 (nB1)	S (1)	S (≤1)	S	None
BTB 02-065 (B1)	S (1)	S (≤1)	R	None
BTB 02-027 (B3)	S (4)	S (≤1)	R	None
BTB 02-012 (B4)	R (8)	S (≤1)	R	None
BTB 02-022 (B3)	R (16)	S (≤1)	R	None
BTB 02-021 (B10)	R (32)	S (≤1)	S	C516T
BTB 02-037 (B3)	R (32)	S (≤1)	R	None
BTB 02-039 (B3)	R (32)	S (≤1)	R	None
BTB 02-070 (B11)	R (64)	R (8)	S	C516T
BTB 02-067 (B2)	R (>256)	R (>256)	R	A1400G

[a] Clinical isolates originating from 10 different patients; designation in parentheses: B- Beijing; nB- non Beijing.
[b] KM, kanamycin; AK, amikacin; SM, streptomycin 4.0 μg/mL; S, susceptible; R, resistant.

Analyses of clinical and sociodemographic data of tuberculosis patients

Paper II. There was no statistically significant relation between the gender or age of the patients and the RFLP patterns of the isolates. In general, there was no clear correlation between RFLP patterns and the geographical origin of the isolates. Thus, the predominant family A was made of clusters which represent patient groups from both the Northern and Southern regions of Estonia (table 7).

Table 7. Characteristics of 209 TB patients and their isolates in relation to IS*6110* RFLP patterns of isolates.

Patients	No.(%)	No of isolates/no of clusters		
		100% similarity	90% similarity	65% similarity[b]
Male	151 (72)	79/32	92/32	81
Female	58 (28)	24/15	28/16	28
Age[a] (mean, 41 yr)				
≤ 41 yr	103 (54)	53/26	63/26	53
> 41 yr	88 (46)	39/26	46/26	44
From:				
North Estonia	140 (67)	65/29	78/29	74
South Estonia	69 (33)	36/22	42/21	35

[a] In 18 cases the ages of the patients were not known.

[b] Number of isolates belonging to family A to E.

Paper IV. *Sociodemographic and clinical data.* The 11 patients with TB could be divided into 2 groups. Group 1 included 6 patients with TB that was characterized by continuous excretion of drug- susceptible *M. tuberculosis* isolates for >10 months, despite anti-TB treatment. Group 2 included 5 patients with TB characterized by continuous excretion of *M. tuberculosis* isolates and a shift from susceptible to resistant strains. The majority of patients in both groups were male (5 of 6 in group 1 and 4 of 5 in group 2). The mean age was 47 years (range, 36 – 67 years), in group 1 and 43 years (range, 29 – 66 years) in group 2 ($P = 0.5$). The majority of patients (all 6 patients in group 1 and 3 patients in group 2) were alcohol abusers ($P = 0.1$). In addition 4 patients in group 1 and all 5 patients in group 2 were unemployed ($P = 0.4$). In both groups, 1 patient had previously been imprisoned. With respect to the manifestation of TB and a history of TB, the patients in the 2 groups did not differ ($P = 0.5$, and $P = 1.0$, respectively) (table 8).

Drug regimens, duration of therapy and patient outcome. Patients in both groups received initial daily therapy in hospital. For group 1, the median duration of treatment was 45 days (range, 18 – 63 days); in group 2, it was 38 days (range, 28 - 52 days) ($P = 0.3$; table 8). The duration of the TB episode varied from 19 to 60 months (mean, 37.4 months) for group 1 and from 14 to 66 months (mean, 42.9 months) for group 2 ($P = 0.5$). There was no difference in the treatment duration between the groups (range, I 5.2 - 19.3 months, and median, 7.6 months for group 1; range, 3.0 - 36.9 months, and median 24.5 months, for group 2; $P = 0.2$) (table 9).

Treatment regimens were regularly changed in both groups. Therapy was changed 3 - 9 times (median, 4 changes) for patient group 1 and 6 - 17 times (median, 7 changes) for patients in group 2 ($P = 0.1$). No standardized regimens were applied during the therapy. In both groups, the daily dosages of drugs used in the treatment regimens were consistent with WHO recommendations (table 9).

The number of treatment interruptions of >2 months during the therapy was similar in both groups (range, 2 - 5 interruptions, and mean, 2.5 interruptions for patients in group 1; range, 2 - 5 interruptions, and mean 3.2 interruptions for patients in group 2; $P = 0.3$); a treatment interruption of >2 months was considered to indicate poor compliance.

The treatment outcome did not differ statistically significantly between group 1 and 2. Three patients in group 1 were cured, and 3 died. One patient in group 2 was cured, 3 patients died, and treatment was stopped for 1 patient because of lack of active drugs remaining in the regimen (the isolate from the patient demonstrated resistance to 9 drugs).

Table 8. Sociodemographic and clinical data for patients with tuberculosis who met the study inclusion criteria.

Group, Patient	Age, years/ sex	Risk factor for TB infection			Manifestation of TB		Previous history of TB	Treatment initiated at hospital	Duration of first hospitali- zation, days	No. of drugs in initial therapeutic regimen	Inclusion of isoniazid and rifampicin in initial therapy
		Unemploy- ment	Alcohol abuse	Prior im- prisonment	Pulmonary	Extra- pulmonary					
1											
1	44/M	Yes	Yes	No	Yes	Yes; pleural	No	Yes	63	2	No
2	36/M	Yes	Yes	No	Yes	No	No	Yes	47	4	Yes
3	50/F	Yes	Yes	No	Yes	No	No	Yes	45	3	Yes
4	47/M	Yes	Yes	Yes	Yes	No	Yes	Yes	42	2	No
5	38/M	No	Yes	No	Yes	Yes; pleural	No	Yes	56	4	Yes
6	67/M	No	Yes	No	Yes	Yes; pleural	No	Yes	18	4	Yes
2											
1	56/M	Yes	No	No	Yes	No	No	Yes	28	2	No
2	29/F	Yes	No	No	Yes	No	No	Yes	27	3	Yes
3	45/M	Yes	Yes	No	Yes	No	No	Yes	52	3	Yes
4	44/M	Yes	Yes	Yes	Yes	Yes; pleural	Yes	Yes	50	4	Yes
5	40/M	Yes	Yes	No	Yes	No	No	Yes	31	4	Yes

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Table 9. Treatment regimens for 11 patients with tuberculosis (TB).

Group, patient	Duration of TB	Duration of	Treatment regimen (duration in months)[a]	Daily dosage of drugs					
				R	H	S	Z	E	K
1									
1	38.3	13.4	HE (2.1)[b]/ HZE (1.7)/HZEK (2.0)[c]/ZEK (0.1)/ZEKR (0.3)/ZK (0.5)/ZE (1.7)[b]/ ZE (3.3)[c]/HZE (0.9)[c]/RZE (0.8)	450/ 600	300/ 450	-	1600/2000	1200/ 1600/ 2000	500
2	30	5.5	HRZS (1.6)/RZ (0.2)[c]/ HRZS (1.0)[c]/HRZES (2.7)	450/ 600	300	1000	1600/2000	1600	-
3	19	5.2	HRS (0.2)/HRZS (1.5)/HRZE (0.5)[c]/HRZE (1.4)[c] /HRZE(1.6)	450/ 600	300	1000	1000/1600/ 2000	1200/ 2000	-
4	60	>9.3	ZE (1.4)/HZE (ND)[c]/ HZE (ND)[c]/HRZE (7.9)	600	300	-	1200/2000	1200/ 1600	-
5	39.9	19.3	HRZS (2.0)[c]/ZE (2.0)[c]/RZE (0.2)/HRZS (1.4)/HRZE (2.0)[c]/HRZ (1.0)[c]/HRZEK (3.2)/HRZE (7.5)	450/ 600	300/ 600	1000	1500/1600/ 2000	800/ 1500/ 1600	1000
6	38.1	5.9	HRZE (0.6)/RZE (2.5)[c]/HRZS (1.1)[b]/HRZS (1.2)[c]/HRZES(0.5)	450/ 600	300	500/ 1000	1500/1600/ 2000	1200/ 1600	-
2									
1[d]	52.9	36.9	RZ (0.6)/HRZ (0.9)/HZ (0.3)/Z (0.3)/ZE (1.5)/ZE (7.6)/HZE (0.1)/ZE (3.2)/HE (0.7)/HZE (3.0)[c]/HZ (2.0)[c]/HRZ (3.9)/HRZE (1.7)/HRZ (0.5)/HRZE (0.9)/ZE (1.2)/ZE (8.2)	600	300/ 600	-	450/ 1000/ 1200/ 1500 /2000	800/ 1200/ 1600	-
2[d]	44	34.3	HRZS (1.8)/HRZE (2.4)[c]/HRZE (2.0)/ HRZE (2.3)[c]/HZ (4.0)/HZ (9.0)/E (1.7)[c]/HZES (4.4)/ZE (6.7)	600	300	1000	2000	1600/ 2000	-
3	38.5	12.4	HRZS (1.7)[c]/HRZ (1.9)/RZ (0.3)[c]/HRE (0.3)[c]/HZE (2.0)[b]/HZE (1.0)[c]/HRZE (1.9)[c]/ZE (3.3)	600	300/ 600	1000	1200/ 1500/ 1600/ 2000	1200/ 1600/ 2000	-
4	13.8	3.0	HRZS (0.4)/RZES (0.4)/HRZE (0.2)/HRZES (1.2)/RZE (0.3)[c]/HRZE (0.5)	600	300	1000	2000	1200/ 1600	-
5[d]	65.6	25.1	HRZS (1.1)[c]/HRE (9.0)/HE (3.7)[c]/HRZE (1.3)/HRZ (0.8)/ZEK (2.8)/ZEK (1.7)[c]/HRZES (2.8)[c]/HZE (1.9)	600	300	1000	1000/ 1200/2000	1200/ 1600	1000

NOTE. E, ethambutol; H, isoniazid; K, kanamycin; ND, not documented; R, rifampicin; S, streptomycin; Z, pyrazinamide.
[a] Each change in treatment is marked by a slash (/); [b] Treatment was stopped for >1 month; [c] Treatment was stopped for >2 months; [d] Second-line drugs were used during the course of treatment but are not included here.

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Paper III. A total of 67 HCW among the 14 730 in the HCW registers were diagnosed as patients with primary TB during the study period. This indicated a mean annual case rate of TB of 91/100 000 HCW. These HCW were employed at 33 healthcare institutions, including 25 hospitals, 7 outpatient clinics and one university institute. Among this group, 23 were physicians, 23 nurses, seven laboratory technicians, 12 assistance nurses and two cleaners (table 10). In 1994 to 1997, the case rates among nurses and laboratory technicians varied between 66 to 82 per 100000, which was over 1.5 times higher than among the general population, which increased during the same period from 41 to 56 cases per 100 000 (figure 11). During the same time period, the case rates among physicians increased from 83 to 147 per 100 000, i.e. rates 1.5 to 2.9 times higher than among the general population. In Tartu University Lung Hospital (TULH), the respective case rates among HCW were higher, reaching the highest rates in 1996 among nurses and laboratory technicians (3450 per 100 000) and in 1998 among physicians (6900 per 100 000) (figure 11). Thus, the risk of TB was almost 30 to 90 times higher among physicians in TULH than in the general population.

Figure 11. Annual case rates of TB among healthcare workers and the general population in Estonia. Case rates among employees of a lung hospital (TULH) with regional responsibility for TB are shown separately.
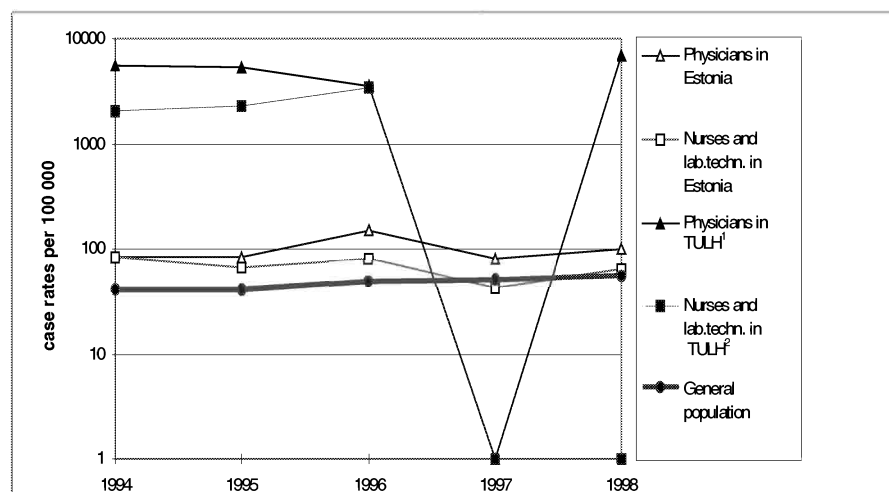
[1]No new cases detected among physician at TULH in 1997.
[2]No new cases detected among nurses and laboratory technicians at TULH in 1997 and 1998.

Table 10. Characteristics of HCW with TB in Estonia in 1994-1998 compared with the general population

Characteristic	HCW with TB 1994-1998 n (%)	Total patients with TB in Estonia*	
		1994 n (%)	1998 n (%)
Total number	67 (100)	518 (100)	820 (100)
Profession			
Physicians	23 (34)		
Nurses and laboratory technicians	30 (45)		
Assistant nurses and cleaners	14 (21)		
Sex			
Female	59 (88)	163 (31)	232 (28)
Male	8 (12)	355 (69)	588 (72)
Ethnicity			
Estonians	49 (73)	NA	540 (66)
Non-Estonians	18 (27)	NA	280 (34)
Site of TB			
Pulmonary	58 (87)	448 (87)	749 (91)
Extrapulmonary	9 (13)	70 (14)	71 (9)
Bacteriological verification			
Smear positive	20 (30)	NA	372 (45)
Culture positive	49 (73)	329 (64)	545 (67)
Smear positive and culture positive	18 (27)	NA	NA
Smear positive and culture not done	2 (3)	NA	NA
Smear negative and culture positive	31 (46)	NA	NA
MDR-TB	18 (38)[†]	24 (10)[‡]	53 (14)[§]

* HCW included.

[†] Susceptibility testing done in 47 cases.

[‡] Susceptibility testing done in 266 cases.

[§] Susceptibility testing done in 377 cases.

NA = no data available; MDR- TB = multidrug-resistant tuberculosis.

Structure of *M. tuberculosis* population in Estonia

In a countrywide study, IS*6110* RFLP patterns of *M. tuberculosis* isolates were remarkably homogeneous, notably among drug resistant isolates (figure 7). For instance 51.6% of the *M. tuberculosis* isolates belonged to five genotype families that share at least 65% similarity in the IS*6110* RFLP patterns (table 5). Of the 209 *M. tuberculosis* isolates 64 (31%) were resistant to one or more of the drugs tested, while 24 (11.5%) of them were multidrug resistant. The majority (87.5%) of all multidrug-resistant isolates, 67.2% of all isolates with any drug resistance belonged to one particular family (family A); classified by spoligotyping as Beijing genotype strains. The other families contained only 2/24 (8.3%) MDR isolates and 10/64 (15.6%) of isolates with any drug resistance (table 5). Among isolates that were not clustered or did not belong to any of the five families, only 1 of 24 MDR (4.2%) and 64 of 145 fully susceptible (44%) isolates were detected.

No statistically significant relationships were noted between the gender or age of the patients and the RFLP patterns of the isolates. Of 33 clusters only six smaller clusters, two or three isolates per cluster, represented patients belonging to the same age group. In general, there was no clear correlation between RFLP patterns and the geographical origin of the isolates (table 7). Thus, the predominant family A was

composed of clusters representing patient groups from both the Northern and Southern regions of Estonia.

However, on a cluster level, a correlation was observed with the geographical origin of the isolates. A comparison of the geographical origin of the isolates in family A shows that the isolates belonging to cluster A_{10} were scattered throughout the community, while all isolates in cluster A_7 and A_{12} were more geographically focused. All of the isolates from cluster A_7 originated from Tartu (Southern Estonia), and all isolates in cluster A_{12} originated from North Estonia.

DISCUSSION

Resistance patterns of *M. tuberculosis*

The first countrywide study carried out in 1994 ascertained that drug resistant TB, and particularly multidrug-resistant TB (MDR-TB), is a serious problem for Estonia. Initial resistance to one or more of the drugs tested was 28%, with 10% being initially multidrug resistant, i.e. resistant to at least isoniazid and rifampicin. These figures were similar to those reported in New York in 1992 (Culliton, 1992). However, they were much higher than in industrial countries neighbouring Estonia, such as Sweden that had a very low rate of initial multidrug resistance of 0.6% in 1994 (The Swedish Tuberculosis Index, 1995).

In a recent countrywide anti-tuberculosis drug resistance survey, a significant upward trend was noted in the prevalence of resistance to any drug among new cases in Estonia, from 28.2 percent in 1994 to 36.9 percent in 1998 (Espinal *et al.*, 2001).
The reasons behind the high rate of drug-resistant strains within the Estonian population have been considered multifactorial. WHO and IUATLD investigators have suggested that the most significant reasons for the continued generation of drug-resistant TB is due to highly irregular anti-tuberculosis treatment and increased circulation of drug-resistant strains due to poor infection control policies (Espinal *et al.*, 2001). Today, molecular epidemiological studies can help identify current and past failures in TB control and allow tracking of the transfer path of the resistant strains (Glynn *et al.*, 1999). Therefore we aimed to examine large numbers of TB cases in Estonia using molecular epidemiological techniques to gain insight into the problem.

The possible contribution of Beijing genotype *M. tuberculosis* strains in the emergence of multidrug-resistant tuberculosis in Estonia

Molecular epidemiology studies of TB in Estonia had not been previously carried out. By using these methods, we surprisingly found a predominant family of *M. tuberculosis* with high homology, involved in TB among Estonian patients. The strains belonging to the predominant family A were clones of *M. tuberculosis*, previously found in China and later on with a lower frequency throughout Asia (Torrea *et al.*, 1995; Lin *et al.*, 1996; Palittapongarnpim *et al.*, 1997). Recent observations suggest that strains of Beijing family possibly share particular phenotypic properties, such as antigens and virulence factors, which may be lead to distinct manifestations in the pathology and epidemiology of TB (Kremer *et al.*, 1999).

The investigation of the structure of *M. tuberculosis* population in Estonia suggests that 1/3 of isolates obtained from our region belong to the Beijing family. Moreover, there was great similarity seen among the MDR *M. tuberculosis* strains selected for molecular analysis in 1997-99 from pulmonary TB patients diagnosed in the Southern and Eastern regions of Estonia. Two major subgroups, with identical or very closely related (similarity >84%) *M. tuberculosis* isolates were identified (Krüüner *et al.*, 2000). These IS*6110* banding patterns were found in 1994 as predominant patterns among the Family A, which included 87% of all MDR strains tested. Thus, the

Beijing genotype family has continued to dominate among new MDR-TB patients in Estonia up to the present day.

Therefore, it is probable that this particular genotype of strains may have influenced the recent steady increase in Estonia in TB morbidity since 1992. Even more importantly, it may have contributed to the emergence of MDR-TB in the region. This is the first countrywide study demonstrating a correlation between the Beijing family and drug resistance. Our findings are consistent with the prolific spread of MDR variants of the "W" (phylogenetic lineage of Beijing family identified in USA) strain in the early 1990s in North America (Friedman *et al.*, 1995, Agerton *et al.*, 1999).

Members of the Beijing genotype group of strains are widely distributed worldwide and have been implicated in several outbreaks in addition to the USA also in Asia, South East Asia and South Africa (Torrea *et al.*, 1995: Lin *et al.*, 1996; Bifani *et al.*, 1999). In the USA, members of the Beijing family have been associated with multi-drug resistance outbreaks as well as with high rates of morbidity and mortality. Most of these outbreaks occurred in institutional settings (Moss *et al.*, 1997; Haas *et al.*, 1998) though the general population was occasionally involved (Bifani *et al.*, 1999; van Rie *et al.*, 1999). Recently a high prevalence of multidrug resistance among Beijing isolates was shown in studies conducted among Russian civilians (Drobniewski *et al.*, 2002) and in prison inmates in Azerbaijan (Pfyffer *et al.*, 2001) and in Russia (Portaels *et al.*, 1999; Drobniewski *et al.*, 2002).

However, it´s success as a pathogen has yet to be understood and is the focus of much research. The question to be answered is if and to what extent Beijing genotype strains have selective advantages over other *M. tuberculosis* genotypes; in their ability to gain resistance and to interact with the host immune defense system. If Beijing genotype strains represent a higher level of evolutionary development of *M. tuberculosis*, selected for as a result of the introduction of tuberculostatics, which inhibit the growth of *M. tuberculosis*, then implications for the treatment of tuberculosis will be highly important (Glynn *et al.*, 2002).

Exogenous reinfection of tuberculosis

Unexpectedly, we managed to show that in five pulmonary TB cases the appearance of drug resistance was noted together with a shift in the *M. tuberculosis* strain. Different studies have indicated that exogenous reinfection may occur following successful treatment (Small *et al.*, 1993; van Rie *et al.*, 1999a; de Boer and van Soolingen, 2000). However, limited data is available regarding the rate at which superinfection will occur during the same episode of TB. Niemann *et al.* have described one TB case with a double infection with both a drug-resistant and a multidrug-resistant strain (Niemann *et al.*, 2000).

Exogenous reinfection with the Beijing family of MDR *M. tuberculosis* was found in all five immunocompetent pulmonary TB patients. In addition, in all these cases, the physicians considered the emergence of drug resistance to be as a result of unsupervised drug administration, poor patient compliance with therapy, and errors in medical prescriptions of drug regimens. The detection of superinfection with a new *M. tuberculosis* strain during the treatment of an episode of active TB is possible only through the use of molecular epidemiology tools. The results obtained using these tools make it clear that patients who are treated in >1 hospital setting can become

infected with a new and more dangerous strain of *M. tuberculosis* if infectious patients are not isolated; on the other hand, it may also be that these particular strains are extremely virulent. This type of nosocomial spread of TB is obscured by the difficulties in diagnosing TB earlier in the course of the disease, and therefore the extent of the problem remains largely unknown.

There have been some reports of exogenous reinfection with multidrug-resistant strains of *M. tuberculosis*, but these were restricted to HIV-positive patients who were immunocompromised (Fine and Small, 1999). However, findings from our study and from the study conducted in 2 poor suburban communities in Cape Town, South Africa (van Rie *et al.*, 1999b), suggest that reinfection may be more common than previously thought and it is not restricted to HIV- positive patients only. These results suggest that in immunocompetent persons living in an area where tuberculosis is endemic, reinfection and progression to active disease may occur any time after treatment has been discontinued or even during treatment for active tuberculosis. Hence, the primary infection does not convey immunity to subsequent infection with an organism of a different genotype. So, there are implications for vaccine development for different genotypes of *M. tuberculosis*.

Tuberculosis as an occupational hazard for healthcare workers

Previously published data indicates that TB infection is more common among health care staff working in high-risk work places (Malasky *et al.*, 1990; Redwood *et al.*, 1993, Ramirez *et al.*, 1992) than among general health care staff (Price *et al.*, 1987; Berman *et al.*, 1981; Vogeler and Burke, 1978). The risk of nosocomial TB among HCW may be evaluated either by the occurrence of clinical disease or by the rate of tuberculin conversions, often regarded as an indicator of TB infection among asymptomatic persons. In Estonia, despite increasing incidence of TB there were no direct investigations into the spread of TB among HCW. Our findings showed that the incidence of clinical TB was 1.5 to three times higher among HCW than in the general population. A chest hospital in charge of regional TB care, had an incidence rate 30 to 90 times higher, and was highest among physicians.

The risk of TB among HCW was substantial in the era before antibiotics, but declined rapidly after 1950 due to decreasing TB incidence in the population and the advent of effective therapy (Menzies *et al.*, 1995). Over the past decade, two factors, both identifiable in Estonia, have profoundly altered views about the risk of TB in HCW. These include 1) the resurgence of TB and 2) the emergence of MDR strains of TB (Menzies *et al.*, 1995). From 1970 to 1989, only two cases of TB were registered among hospital employees in the Tartu University Lung Hospital, while 18 cases of pulmonary TB were diagnosed between 1994 and 1998. In the same chest hospital a very high proportion (72%) of the employees with TB were infected with MDR strains. No known risk factors for TB other than increase in occupational exposure could be identified for these people. All of the HCW with TB tested negative for HIV. Among all culture confirmed TB cases in HCW, eighteen (38%) had MDR-TB. Our data illustrate that TB in HCW is often MDR now that MDR-TB has become more common in the general population. Thus, the prevention of nosocomial spread of TB is particularly important when MDR-TB is being exposed in the hospital environment.

Hence, it is clear that the risk of nosocomial tuberculosis will continue as long as health care is provided to patients with active TB (Menzies *et al.*, 1995). The objective of infection control plans for Estonia should focus on reduction of this risk to as low as possible. Therefore, the development of guidelines to ensure safety has become an important issue. For TB infection control (IC) an acting institution could be constituted having clear responsibilities and appropriate rights for co-ordination and inspection. In the present scenario, the TB IC plan could possibly be assigned to an IC administrator for TB within the Inspection of Health Care. For preventing HCW they should have the authority to develop and set strict IC policies. Also, close co-operation with the Estonian National Reference Laboratory is required for detection and confirmation of outbreaks by using advanced molecular tools.

According to experience in the USA, prevention among HCW may be approached in the following context; early identification, prompt initiation of effective chemotherapy, physical isolation, dilution of the air to limit the concentration of infectious particles, ultraviolet irradiation of the air to reduce the number of viable bacilli, physical filtration to remove bacilli from the air, and respiratory protective devices to filter out infectious particles from inhaled air (Iseman, 2000).
Also, HCW must be trained to understand the epidemiology of TB: the potential for occupational exposure and the principles of infection control measures.
In addition, developed recommendations should be applicable in real-life, sustainable, cost-effective and compatible with a humane, professional environment for care (Nolan, 1994).

Persistence of isogenic drug-susceptible *M. tuberculosis* strains in patients with treatment failure of active pulmonary tuberculosis

We aimed to investigate the means by which drug resistance evolves among drug-susceptible *M. tuberculosis* strains during antiTB treatment. We examined a retrospective case series including patients with TB who had experienced failure of treatment for active pulmonary TB and who initially had received diagnosis of infection with drug-susceptible *M. tuberculosis*. We have followed up 11 patients selected from patients in the ENRL TB register whose treatment failed during a 6-year period. We aimed to obtain at least three sequential isolates from the same TB episode from each patient and to maintain an interval between isolates of ≥2 months. We found that 6 patients excreted isogenic drug-susceptible *M. tuberculosis* strains for >10 months. In contrast, in 5 patients a strain shift occurred concurrently with the appearance of multidrug resistance.

According to Mitchison, treatment failures among patients who were infected initially with drug-susceptible bacilli are rare among patients who have been treated with regimens of ≥3 drugs that include rifampicin (Mitchison and Nunn, 1986). In contrast to those findings, our study has demonstrated that viable organisms can persist in sputum for several months or years (as demonstrated by culture positivity during therapy), despite demonstration of drug susceptibility *in vitro* and administration of appropriate doses of drugs for chemotherapy. However, this persistence was associated with marked irregularity of TB therapy.

Mitchison has described the emergence of drug resistance during short-course chemotherapy with multiple drugs, solely due to irregularity in administration of drugs, because of the occurrence of several cycles, selection of mutants that are

resistant, relative to the susceptible bacterial population, is possible (Mitchison, 1998). However, the drug-susceptible *M. tuberculosis* strains obtained from 6 patients in our study did not develop resistance, despite administration of highly irregular therapy. This might be the result of longer intervals during which the treatment was stopped; thereafter, during the regrowth period, the proportion of susceptible bacilli could quickly increase again.

On the basis of our investigation of sequential isolates obtained from patients who experienced failure of treatment for active pulmonary TB, we propose that highly irregular treatment and unfavorable sociodemographic factors do not always lead to the emergence of drug resistance in drug-susceptible *M. tuberculosis* strains, even during a long treatment period. Regardless, the host did not benefit from the drug susceptibility of infecting *M. tuberculosis*; one-half of our patients died.
It has been demonstrated in our co-operative research with CDC that TB patients with MDR-TB are more likely than those with drug susceptible TB to have treatment failure (Lockman *et al.*, 2001). Nevertheless, six patients in this work were classified as treatment failures with drug susceptible TB and had unsuccessful treatment outcome.

However, little is known about the range and diversity of the host genes involved in tuberculosis susceptibility and how genetic variability and racial diversity affect immune responses to *M. tuberculosis* (Schurr, 2001).

Also, it seems that the drug susceptible *M. tuberculosis* strains are not evolving drug resistance *in vivo* as easily as it was believed to happen. Yet, the scenario might be very different for the heterogenic population of bacteria, e.g. where the drug resistant mutants are already existing and are ready for subsequent selection and multiplication. So, the need for further studies is great.

Drug resistance to second-line and alternative drugs

Multidrug-resistant tuberculosis (MDR-TB), defined by isolates of *Mycobacterium tuberculosis* resistant to isoniazid and rifampicin, is a significant public health problem in several countries, particularly those that comprised the former USSR (Drobniewski *et al.*, 1996; Pablos-Mendez *et al.*, 1998; Coninx *et al.*, 1998). Laboratories supporting TB control programs in populations of these countries with endemic MDR-TB are increasingly required to provide rapid, reliable drug susceptibility testing (DST) not only for the first-line drugs (i.e., isoniazid, rifampicin, ethambutol, and streptomycin) but also for second-line and alternative drugs (e.g., amikacin, capreomycin, ciprofloxacin, clarithromycin, clofazimine, cycloserine, ethionamide, p-aminosalicylic acid, thiacetazone). Accurate laboratory antimicrobial susceptibility testing data of second-line drugs will support clinical decision-making and help prevent the emergence of further drug resistance in patients with MDR-TB (WHO/CDS/TB2001.288). Also, the knowledge of regional homogeneity or individual heterogeneity of antimicrobial susceptibility pattern of MDR *M. tuberculosis* to second-line and alternative drugs is required for applying an appropriate chemotherapy for MDR-TB treatment in Estonia.

Unfortunately, the protocols for performing second-line DST are not standardized and the recommended critical concentrations for various media are often based on scattered small-scale studies (Casal *et al.*, 1988; Chen *et al.*, 1989; Fenlon *et al.*,

1986; Heifets and Lindholm-Levy, 1989; Heifets *et al.*, 1991b; Lee and Heifets, 1987) including only on a narrow spectrum of drugs and/or tested only a rather limited number of *M. tuberculosis* strains. More recently collaborative efforts have been made to optimize the methods for performing DST for second-line and newer antimicrobial agents on solid media and by the radiometric BACTEC method (Pfyffer *et al.*, 1999).

In the present study the 182 MDR isolates of *M. tuberculosis* had a high degree of clonal origin demonstrated by IS*6110* RFLP (data not shown). They were also found to have similar drug susceptibility patterns to primary, second-line and newer antimicrobials throughout the 3 years period. Thus antimicrobial susceptibility pattern of MDR *M. tuberculosis* isolates from clonal origin can be stable. In a previous study 87% of all Estonian MDR strains were classified by spoligotyping as Beijing genotype (Krüüner *et al.*, 2001). It is interesting to note that the antimicrobial susceptibility among MDR-TB isolates from newly diagnosed patient did not differ from re-treatment patients, of whom many have had earlier therapy for MDR-TB. This indicates that the problem with resistance to second-line drugs is more related to the spread of already resistant strains rather than a broad development of extended drug resistance during the therapy.

Clofazimine, capreomycin, clarithromycin and ciprofloxacin were most active; amikacin and ethionamide showed a smaller extent of activity, while azithromycin, cycloserine, p-aminosalicylic acid and thiacetazone showed only marginal or no *in vitro* effect in our assay. The results, especially to four last drugs, should be interpreted carefully since there are methodological limitations. The further need for research regarding how to best perform *in vitro* susceptibility testing of newer second-line drugs, and the clinical value of such tests, is clear.

More than 70% of all MDR *M. tuberculosis* isolates included to this study were susceptible at least to 6 second-line or newer antimicrobial agents. Based on these results, in 2/3 of MDR-TB cases a standardized treatment regimen with 6 II line drugs can be suggested. Thus, for 30% of cases an additional testing of an extended panel of drugs is needed in a situation where >70% of all MDR *M. tuberculosis* clinical isolates are resistant to all 4 first line drugs.

MDR *M. tuberculosis* clinical isolates with unusual kanamycin-resistant but amikacin-susceptible phenotype

Numerous studies have reported cross-resistance between the two (closely related) deoxy- streptamine aminoglycosides kanamycin and amikacin (Heifets, 1991a; Sutton *et al.*, 1966; Alangaden *et al.*, 1998; Riska *et al.*, 2000). In our study, however, the phenotype of kanamycin-resistant but amikacin-susceptible *M. tuberculosis* was found at an unexpected high frequency among MDR *M. tuberculosis* clinical isolates in Estonia (>50% of isolates tested). This finding was not in agreement with earlier reports and so therefore it was of interest to determine the nature of molecular mechanism involved.

A mutation possibly related to an intermediate level of resistance (MICs >32 and ≤64 µg/mL) to kanamycin was identified in two *M. tuberculosis* isolates selected for sequencing of the whole *rrs* gene. To our knowledge this mutation, showing a

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thymine for cytosine substitution at the 16S rRNA position 516, has not been previously associated with kanamycin resistance in *M. tuberculosis*. This mutation has been reported in streptomycin resistant strains (Dobner *et al.*, 1997; Ramaswamy and Musser, 1998), which is in conflict with our findings since our strains were KM-resistant but SM-susceptible.

Moreover, Victor *et al.* have proposed that nucleotide change (C-to-T transition) at position 491 of the *rrs* gene (close to the position where we found the thymine for cytosine substitution) is a polymorphism that is not associated with drug resistance (Victor *et al.*, 2001). Nonetheless, in two earlier studies this mutation has been identified as encoding resistance to streptomycin (Dobner *et al.*, 1997; Ramaswamy and Musser, 1998). All of these contradicting findings highlight the importance of establishing the causal relationship between any given mutation and actual drug resistance.

We did not found any mutations at the 1400, 1401 and 1483 positions in any of the 40 KM-resistant and AK-susceptible MDR *M. tuberculosis* clinical isolates tested. This is in agreement with earlier reports where no mutations in this region were found in low-level (MIC ≤4-32 µg/mL) AK-KM cross-resistant *M. tuberculosis* (Alangaden *et al.*, 1998). Nucleotide substitutions at 1400 in the *rrs* gene may be used as an important marker of high-level AK-KM resistance (Alangaden *et al.*, 1998; Suzuki *et al.*, 1998; Taniguchi *et al.*, 1997). Also in our study the high level AK-KM cross-resistance was confirmed by the presence of a mutation in the 1400 region.

So far genetic methods fail to detect all clinically relevant levels of drug resistance to aminoglycosides. Consequently, it is important to test antimicrobial susceptibility of clinical isolates of *M. tuberculosis* by culture. Since amikacin-kanamycin cross-resistance is not present in all strains, we propose that clinical isolates should be tested to both of the drugs simultaneously.

Prospects of tuberculosis control and future considerations

At the turn of the millennium no continent can claim victory over the tuberculosis epidemic. Furthermore, it is increasingly realized that no single country can eliminate tuberculosis (Arnadottir, 2001).
Tuberculosis, which has ravaged humankind since Neolithic times is easily transmitted by aerosol droplets from person to person. It is this easy mode of transmission that most likely explains the persistence of tuberculosis as one of the most pressing health problems of today (Schurr, 2001).

However, where to place blame for tuberculosis transmission – the host or pathogen? Is the transmission due to biological host factors (location and type of disease) coupled with social host factors such as homelessness and substance abuse? Or could it be that certain *M. tuberculosis* strains, such as the Beijing strain, have unique traits that differentiate them from other isolates? Salamon and co-workers identified a correlation between the number of genetic deletions in *M. tuberculosis* and the risk for cavitary tuberculosis among residents of San Francisco (Salamon *et al.*, 2000). Houston outbreak strain known as HN878 associated with hypervirulence elicits altered cytokine profiles in murine tuberculosis compared with standard strains (Manca *et al.*, 2001). The possibility of altered lipid antigens in this strain is also being explored. These data suggest that bacterial genotype may predict disease phenotype.

On the other hand according to Sepkowitz *et al.*, there are several reasons for increase in the incidence of tuberculosis: the deterioration of public health care facilities, the rise in the number of homeless persons and persons living in congregate settings, the continued influx of immigrants from countries where tuberculosis is endemic, and the emergence of the AIDS epidemic (Sepkowitz *et al.*, 1995).

However, in the 21st century there are at least two main forces counteracting control measures for tuberculosis: HIV in Africa and multidrug resistance of *M. tuberculosis* in parts of Europe and the former Soviet Union (Arnadottir, 2001).

The last decade of the twentieth century saw an increasing incidence and prevalence of tuberculosis in many Eastern European countries, including the three Baltic republics. Of the three Baltic countries, the rate of tuberculosis is lowest in Estonia. After a steep increase in the rate of tuberculosis since 1990, the statistics from 1998-2002 suggest that a plateau (at a rate of approximately 50 per 100 000 inhabitants) may have been reached. So far, the HIV epidemic has had very little influence on TB epidemiology in Estonia. Altogether 27 HIV and TB co-infected cases have been diagnosed since 1997. Because of the high risk of developing clinical TB in HIV-infected persons, by either endogenous reactivation or de-novo infection, the recent increase in case load of HIV from 0.65 per 100 000 in 1999 to 66 in 2001 will put additional strain on national TB control program. TB is the only HIV-associated respiratory infection that is easily transmitted via the respiratory route among persons with HIV infection and from them, to non-HIV infected persons.

To grasp the scope of the problem of MDR-TB in Estonia the following activities could be considered:

1. Application of advanced molecular methods for three main purposes in TB control:
   - early diagnosis of MDR-TB among all suspects by using e.g. line probe assay for the detection of mutations in the *rpo*B gene
   - detection of transmitters of *M. tuberculosis* by IS*6110* RFLP typing
   - identification of exogenous re-infection versus generation of drug resistance of *M. tuberculosis* as a possible reasons of treatment failure

2. For epidemiological purposes invention of the full pool of MDR-TB transmitters (backlog of previously treated TB patients- e.g. "chronic TB cases") is very important. The most valuable data will be obtained if in addition to conventional contact tracing, the molecular epidemiology tools will be employed as well.

3. For preventing the possible onward (MDR) TB epidemic and for sharper focusing of actual interventions, the "overlap"of groups at risk for HIV and TB should be identified.

4. Although, the DOTS is now fully expanded in Estonia, there is need for the further strengthening of quality assurance, in particular regarding case management and recording, along with the employment of molecular epidemiological methods when necessary.

CONCLUSIONS

- High rates of initial drug resistance were detected among pulmonary isolates of *M. tuberculosis* during the first countrywide study in Estonia in 1994: 28% were resistant to one or more of the drugs tested, with 10% being multidrug-resistant. This places Estonia among the countries with the highest rates of multidrug-resistant TB in the world.

- Molecular typing with IS*6110* RFLP has revealed that 29% of Estonian *M. tuberculosis* isolates belonged to the genetically closely related group of strains, family A, with a predominant IS*6110* banding pattern. The isolates of family A were classified by spoligotyping as Beijing genotype strains, widely found in Asia.

- The Beijing genotype encompassed the majority (87.5%) of all multidrug-resistant isolates and two thirds (67.2%) of all isolates with any drug resistance. Thus, the limited number of genetically, closely related *M. tuberculosis* clones might have contributed substantially to the emergence of drug resistant TB throughout Estonia. This is the first countrywide study demonstrating a correlation between the spread of Beijing family in the population and increased drug resistance.

- The incidence of TB among health care workers (HCW) in Estonia was 1.5 to 3 times higher (mean 91/100 000/year) than in the general population. In a chest hospital, the incidence was 30 to 90 times higher. The highest rate was observed among physicians, demonstrating their failure to perceive vulnerability to *M. tuberculosis*. More than 1/3 (38%) of all *M. tuberculosis* isolates obtained from HCW were multidrug-resistant.

- The nosocomial isolates of *M. tuberculosis* can often be MDR after MDR- TB becomes more common in the general population. Hence, the control of nosocomial spread of TB is particularly important in Estonia, where a high incidence of both TB and MDR-TB is found.

- Initially drug-susceptible *M. tuberculosis* does not always develop drug resistance even despite highly irregular and prolonged therapy. However, the sustained susceptibility of *M. tuberculosis* does not grant treatment success.

- When advanced methods of molecular typing are not employed, exogenous re-infection with drug resistant *M. tuberculosis* may be misinterpreted as creation of drug resistance due to irregular chemotherapy.

- In Estonia, a standardized treatment regimen with up to 6 second-line drugs can be suggested for 2/3 of MDR-TB patients. For the remaining cases additional testing to an extended panel of drugs would be needed to identify active drugs for individualized therapy.

- The radiometric Bactec 460 procedure is a simple, reproducible and rapid method requiring 6-10 days on average to generate antimicrobial susceptibility testing results to amikacin, capreomycin, ciprofloxacin, and ethionamide. The results for other drugs should be interpreted carefully since

there are methodological limitations. Further studies are needed to increase our understanding regarding both methodology and the clinical relevance of such *in vitro* testing.

- As kanamycin-amikacin cross-resistance is not present in all *M. tuberculosis* clinical isolates, all resistant isolates should be tested to both drugs simultaneously.

- To date, genetic methods fail to detect all clinically relevant levels of drug resistance to aminoglycosides therefore it is important to test antimicrobial susceptibility of clinical isolates of *M. tuberculosis* by culture.

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