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**MOLECULAR CHARACTERIZATION OF
MYCOBACTERIUM TUBERCULOSIS
COMPLEX ISOLATES FROM SWEDEN**

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“tuberculosis will not be eliminated anywhere until it is eliminated everywhere”
(Donald Enarson)

ABSTRACT

Tuberculosis (TB), caused by the *Mycobacterium tuberculosis* complex (MTC), is a serious infection in man and animals. According to the World Health Organisation (WHO), about one third of the world's population is infected with this organism, around nine million new cases are registered every year and two million people die from the infection. Because of the failure of TB treatment regimens, drug resistant (DR) and multi-drug resistant (MDR) TB are increasing. At present, Sweden is one of the countries with the lowest incidence of TB in the world, the majority of TB patients coming from countries with a high incidence of TB.

The current study aimed at investigating the prevalence and transmission of DRTB in Sweden. In the first part of the study 400 DR strains isolated from 315 foreign-born patients and 85 Swedish-born patients from 1994-2005 were analysed using the molecular techniques restriction fragment length polymorphism (RFLP) and spoligotyping. The isolates were resistant to at least one of the first line drugs; streptomycin (SM), isoniazid (INH), ethambutol (EMB) or rifampicin (RIF). Fifty one percent (203/400) of the isolates were clustered by RFLP in 35 different clusters. The size of the clusters varied from 2 to 96 isolates. Most of the clusters comprised only two to three individuals except one large cluster that consisted of 96 patients harbouring isolates with an identical RFLP pattern. The majority of the patients in this cluster were from East Africa, but during the later part of the study several Swedish-born patients infected with this strain appeared.

In the second part of the study the presence and transmission of DR MTC isolates of the Beijing genotype was investigated. The Beijing genotype is a distinct genetic lineage, which has caused substantial transmission of disease in many regions of the world. Seventy DR isolates of the Beijing genotype, 17 of which were MDR and one extensively drug resistant (XDR) were identified during 1994-2008. The majority of the patients came from countries where the Beijing lineage is prevalent. Using different genotyping methods (IS6110 and IS1547 RFLP, spoligotyping, *Rv1335* gene polymorphism, regions of difference RD105, RD142, RD150, RD181 and mutations in *mutT2*, *mutT4* genes) the 70 isolates were allocated to 11 different genetic sublineages. The majority of the isolates (54/70) belonged to the typical "modern" Beijing lineages. Ten clusters of Beijing strains were found, the largest cluster comprising five patients. Contact tracing only established epidemiological linkage between two patients. Thus, there is no extensive spread of the Beijing genotype in Sweden.

These studies illustrate that molecular characterization of DRTB strains offers a powerful tool to trace and monitor transmission as well as to identify deficiencies in national TB control programs.

LIST OF PUBLICATIONS

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LIST OF ABBREVIATIONS

AFRI	Africanum
AIDS	Acquired Immuno Deficiency Syndrome
BCG	Bacille Calmette-Guerin
C	Cytosine
CAS	Central Asian Strain
DNA	Deoxyribonucleic Acid
DR	Drug Resistant
DST	Drug Susceptibility Testing
EAI	East-African-Indian
EMB	Ethambutol
G	Guanine
IFN- γ	Interferon-gamma
INH	Isoniazid
H	Haarlem
HIV	Human Immunodeficiency Virus
IS	Insertion Sequence
LAM	Latin-American-Mediterranean
MDR	Multi-drug Resistant
MIRU	Mycobacterial Interspersed Repetitive Units
MTC	<i>Mycobacterium tuberculosis</i> complex
PAS	Para-aminosalicylic Acid
PCR	Polymerase Chain Reaction
PPD	Purified Protein Derivative
PZA	Pyrazinamide
RNA	Ribonucleic Acid
RIF	Rifampicin
RD	Region of Difference
RFLP	Restriction Fragment Length Polymorphism
SIT	Spoligotype International Type
SM	Streptomycin
TB	Tuberculosis
TCH	Thiophen-2-carboxylic Hydrazide
UPGMA	Unweighted Pair-Group Method using Arithmetic Averages
WHO	World Health Organization
XDR	Extensively Drug Resistant
ZN	Ziehl-Neelsen

1 INTRODUCTION

Tuberculosis (TB), caused by the *Mycobacterium tuberculosis* complex (MTC), is a serious infection in man and animals. Analyses of ancient DNAs of MTC found by archeologists from old graves as well as from ancient mummified bodies of Egyptians traced back the existence of TB as far back as 5000 years [1]. TB, is an old disease that has infected different populations around the world and it is considered as one of the most successful human and animal pathogen. Even though TB today in most cases is curable and in spite of the availability of effective antibiotics for the treatment of the diseases, it still remains as a major global health problem. According to WHO studies, about one third of the world's population is infected with this organism, around 9.27 million new cases were estimated worldwide in 2007 which showed an increase from 9.24 million in 2006 [2]. Every year around two millions people die from infection with TB.

TB has been shown to be increasing in many countries around the world. According to De Backer [3] the rise is mainly due to a global TB elevation in developing countries, the enlarged number of patients suffering from human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) worldwide, an increasing number of elderly patients and the emergence of multidrug resistant (MDR) TB. It has recently been shown that the largest global burden of TB occurs mainly in two regions of the world that is Asia, which accounts for 55% and Africa which accounts for 31% [4].

Because of the failure of TB treatment as well as poor drug management, drug resistant (DR) and multidrug-resistant tuberculosis (MDR) TB in combination with HIV co-infection has now complicated TB control worldwide specifically in sub-Saharan Africa and Asia. Most of the TB cases in Sweden are imported via immigrants that come from areas with a high incidence of TB. I have characterized DR MTC isolates using different molecular techniques to monitor the transmission pattern of DR MTC isolates from Swedish patients.

1.1 HISTORY OF TUBERCULOSIS

TB caused by *Mycobacteria* species is an old disease which was recorded in many human histories. Almost all ancient civilizations describe TB in their old scripts and its existence has been traced in prehistoric skeletal remains. TB was the first ancient infectious disease detected by the DNA of the causative bacteria and it was assumed widely geographically distributed [5].

The earliest populations studied were from ancient Egypt [1], from the "Tombs of the Nobles" followed by the study of pre-Columbian TB in the Northern Chile [6]. Molecular evidence has identified *M. tuberculosis* as the cause of lesions in 5000-year-old mummies from Egypt. Analysis of old bones found submerged off the coast of Israel, at Atlit-Yam, provided genetic evidence for the earliest known cases of TB around the Middle East region, dated 9000 years old [7]. But the oldest TB was traced

to prehistory back to the Pleistocene, from the bison metacarpal material found at the Natural Trap Cave in Wyoming, USA that according to stratigraphy and C¹⁴ dating, was 17,500 years old [5].

TB grew to epidemic proportions in Europe from the 16th century on because of the growing population, the industrialization and development of large urban settings [8]. As a result of this the population densities increased and created ideal conditions for the spread of TB. Crowded housing was an important factor in facilitating the spread of TB, especially when TB patients shared bedrooms with other persons or family members including children. As a result, TB came to be the leading cause of death in Europe in the first half of the 19th century [8]. In 1882, Dr Robert Koch identified an acid fast bacterium, *M. tuberculosis* as the causative agent of TB [9].

The disease then began to decline in developed countries from the mid-20th century. The history of TB changed with the introduction of streptomycin (SM) and subsequent development of other anti-tuberculosis agents. Drug treatment is fundamental for controlling TB, it promotes the cure of patients and breaks the transmission chains when properly administered and the treatment regimen is completed. The main reasons for the TB decline were the development of diagnostics, effective chemotherapy, vaccination with Bacille Calmette-Guérin (BCG) as well as improvement of the life standard, nutrition, housing, sanitation and the environment [8]. But TB is still increasing in developing countries especially in Asia and Africa. Approximately 95% of new cases and 98% of deaths occur in developing nations [10], where HIV infections are common, generally due to the few resources available to ensure proper treatment. In 1993 WHO declared TB as a global emergency even though the initial response from the international community was sluggish and inadequate [11].

1.2 GLOBAL BURDEN OF TUBERCULOSIS

Tuberculosis is a major global health problem with more than 9 million new cases and 2 million deaths reported in 2007 alone [2]. The burden of TB falls mainly on the developing nations where 95% of cases and 98% of deaths occur [10]. Most of the TB cases are from countries, where there are inadequate facilities for the diagnosis and sporadic supply of drugs for treatment of the disease.

Despite the fact that effective drugs to treat the disease have been available the last 50 years, the global epidemic is growing and the spread of HIV, the emergence of DRTB and the breakdown of public health services are adding to the impact of the disease. In many countries of Africa, civil war, famine, alcoholism, tropical diseases, internal displacement and poverty have contributed to the ineffectiveness of TB control programs. Presently, the global TB control effort is threatened by failures in the countries in Southeast Asia, Africa and the former Soviet Union. After the dissolution of the Soviet Union some countries experienced poorly functioning TB control programs due to the breakdown of the health care infrastructure [12]. In Africa and Asia the increase in TB could be associated to the aggravation by the HIV epidemic over the last years. Large numbers of untreated patients in a society could act as a

reservoir or base for the infection and could lead to the increment of the TB incidence. Untreated persons with active TB can infect an average of 10-15 other people every year [13].

According to the WHO globally, the incidence rate of TB is 139/100,000 but in the African region the incidence rate is 363/100,000 compared to the United States case rate of 4.4/100,000 [12, 14]. MDR TB is very low in developed countries, but it is becoming a serious problem in some parts of the world where incomplete treatment and treatment without drug susceptibility testing is common. By and large, the global burden of TB remains enormous because of poor TB control as well as high rates of TB and HIV co-infection in countries with poorly functioning TB control programs.

1.3 TUBERCULOSIS IN SWEDEN

Mortality due to TB was very high in Europe during the first half of the 19th century [8]. Even though TB was considered as a poor people's disease it has left some scars in the noble families of Sweden, for example, the brother of King Oskar II died from TB in 1852 at the age of 25 [15]. In 1940 the morbidity and mortality due to TB was very high in Sweden, estimated at 289/100,000 population and 70/100,000 respectively [16]. Since the mid-20th century the morbidity and mortality due to TB has decreased significantly in Sweden. The TB declination in Sweden was due to many factors, some of them are:

1. BCG vaccination which started in 1927 [17]
2. Introduction of SM and isoniazid (INH) chemotherapy in combination with para-aminosalicylic acid (PAS) which started in 1944
3. Better control of alcoholism in the society
4. Improvement in housing and hygiene
5. Pasteurization of milk and dairy products which started in 1939 [18]
6. Separation of patients with open TB and their treatment in sanatoria; which were based on rest, fresh air and good nutrition.
7. Eradication of *M. bovis* from cattle in 1958 [19]
8. Successful detection and treatment of the disease, including the development and improvement of laboratory facilities and the training of personnel.
9. Adequate attention and the political commitment as well as the social action to stop the spread of disease.

In 2003, the number of new cases of TB notified were 410 (4.6/100,000 population), the lowest number ever registered. TB morbidity and mortality rates in the Swedish-born population have been among the lowest in the world for some decades, and the goal of eradication seems nearer than in most other countries, but "tuberculosis will not be eliminated anywhere until it is eliminated everywhere" (Donald Enarson, the Union against Tuberculosis and Lung Disease). In 2009 the number of new TB cases increased to 643. An increase was seen among foreign-born persons who constituted 82% of new TB cases in 2009. As the TB incidence decreased in the Swedish-born population there was an increment in incidence in the foreign-born

population. The majority of the foreign-born patients were immigrants that came from countries with a high burden of TB [20-22].

1.4 MYCOBACTERIUM TUBERCULOSIS COMPLEX (MTC)

Human TB is caused by members of the MTC, which is comprised of a closely genetically related group, with a high guanine cytosine (GC) content. However, they differ in host range and pathogenicity. The MTC contains *M. tuberculosis*, *M. africanum*, *M. canettii* where the natural host is humans, whereas *M. bovis*, *M. caprae*, *M. microti* and *M. pinnipedii* usually have animals as their natural hosts. There are numerous reports on TB patients infected by *M. bovis*, especially old Swedish and British natives as well as other Europeans due to close contact with domestic animals and consumption of non pasteurization milks [19, 23]. Furthermore there are other reports describing animals infected with *M. tuberculosis* [24]. Therefore it is important to differentiate the MTC members for the accurate diagnosis of mycobacterial disease, public health surveillance and from an epidemiological point of view.

1.4.1 Mycobacterium tuberculosis

M. tuberculosis is a human pathogenic bacteria belonging to the genus Mycobacteria, and is the major causative agent of human TB. This pathogen was first isolated by Robert Koch in 1882 [9] and is the most successful bacteria which existed over 5000 years ago and has infected almost one third of the population of the world. Earlier it was thought that *M. tuberculosis* has been derived from *M. bovis* and adapted to the human host. However after comparison of the whole genome sequence of *M. tuberculosis* and the other members of the MTC, including *M. bovis*, it was found that *M. bovis* was the one with the most regional genomic deletions. Out of these analyses it was postulated that *M. tuberculosis* could not have been derived from *M. bovis*, but on the contrary that *M. bovis* has descended from *M. tuberculosis* [25, 26]. Phenotypically, *M. tuberculosis* can be easily identified using analyses such as nitrate reductase, production of niacin and resistance to thiophen-2-carboxylic acid hydrazide (TCH) and sensitivity to pyrazinamidase [27, 28].

Genotypically, using spacer oligonucleotide typing (spoligotyping) *M. tuberculosis* has been further classified into different phylogenetic lineages [29]. These lineages are spread around the world and demonstrate differences in their geographical distributions and concentrations to certain populations [30].

1.4.2 Mycobacterium bovis

Bovine TB is a zoonotic disease with potential public health and socio-economic significance as it can affect international trade in animals and animal products. The primary sources of infection for humans are consumption of unpasteurized milk and close association between humans and infected animals. Global prevalence of human

TB due to *M. bovis* has been estimated at 3.1% of all TB cases and most of the infections are extra-pulmonary TB cases with rare cases of pulmonary TB. In industrialized countries, the incidence of TB due to *M. bovis* in humans is almost at zero level as a result of pasteurization of milk and animal products and eradication of bovine TB in cattle populations [31].

Australian investigators, based on the frequency of bovine TB cases diagnosed, had suggested in 1989, that bovine TB should be considered as an occupational hazard in abattoir workers, where the disease is transmitted by inhalation causing pulmonary TB [32]. In industrialized countries, human TB cases due to *M. bovis* are relatively rare because of TB control in cattle. Nevertheless, it is estimated that in less than 1% of all reported TB cases the disease is caused by *M. bovis*. This is probably due to reactivation of dormant lesions among the elderly [23]. On the contrary, rural and some urban dwellers in Africa still consume unpasteurized and sour milk potentially infected with *M. bovis*. In many African countries bovine TB is common in both cattle and wildlife but control policies have not been enforced due to cost implications, lack of capacity, and infrastructure limitations [33]. Sweden was declared free from *M. bovis* in cattle in 1958 [19].

M. bovis can be distinguished from *M. tuberculosis* easily on the basis of epidemiology, phenotype and by some genetic markers. *M. bovis* does not produce niacin, does not reduce nitrate and is sensitive to TCH but resistant to pyrazinamide (PZA) [28]. There is also a non-virulent strain of *M. bovis* called Bacillus Calmette Guerin (BCG), which was descended from a virulent isolate of *M. bovis* by subculturing on glycerin-bile-potato mixture for 230 passages. BCG has been used since 1921 as a vaccine to immunize humans against TB in many countries of the world [34]

1.4.3 *Mycobacterium microti*

M. microti was first identified from a field vole in 1937 by Wells [35]. It is mainly found in small rodents as voles, wood mice and shrews. But *M. microti* has also been detected in domestic animals such as pigs, cat, dogs, cows and even in humans [36] [37-39]. It is a curved acid-fast bacillus, earlier detected only in immunocompromised individuals, but later on reported to cause disease in immunocompetent individuals [35, 40, 41]. In early studies this pathogen was considered as a naturally attenuated non-pathogenic mycobacterium and was used as vaccine in two European countries. Firstly it was used in UK where more than 10,000 people were vaccinated from 1946-1961 [42] and secondly in Czechoslovakia from 1951 to 1969 where about 500,000 children were vaccinated [43]. Primary isolation of this bacterium is difficult since it grows poorly on traditional solid egg media. The slow growth rate, about 8-12 weeks, makes it difficult to differentiate from other species of MTC using biochemical tests. This might also be the reason why studies on humans have not been carried out outside of Europe. Only one case has so far been reported from Africa. *M. microti* was there isolated from rock hyrax, and the spoligotype pattern was different from those reported from the UK and the Netherlands [44].

M. microti gives a negative response to purified protein derivative (PPD) skin test and interferon-gamma (IFN- γ) release assays [35].

1.4.4 *Mycobacterium africanum*

M. africanum was first detected and isolated in 1968 from sputum of a Senegalese patient that suffered from pulmonary TB [45]. Earlier, *M. africanum* has been identified by biochemical characterization having an intermediate position in between *M. tuberculosis* and *M. bovis* [46, 47]. Strains named *M. africanum* have been divided in two subtypes; I and II, but presently only the *M. africanum* subtype I, geographically originating from West Africa, is regarded as genetically different from *M. tuberculosis*, while the *M. africanum* subtype II, from East Africa, is regarded as a specific sublineage of *M. tuberculosis* [48]. Recent studies show differences in distribution of *M. africanum* in West African countries, with the highest prevalence (50%) in Guinea-Bissau [46]. *M. africanum* (subtype I) can be distinguished from the rest of the MTC by genomic deletion analysis of region of difference RD9 and by spoligotyping where it lacks spacer 7-9 and spacer 39 [47, 49].

1.4.5 *Mycobacterium canetti*

M. canetti was first isolated in 1969 by Georges Canetti from a French farmer. Later on, *M. canetti* was isolated in 1997, when van Soolingen reported a case of lymph node TB in a two year old Somali child [50, 51]. The year thereafter, Pfyffer described abdominal lymphatic TB in a 56-year-old Swiss man who lived in Kenya [52]. In 1998 and 1999 *M. canetti* was isolated from sputum from two soldiers who had stayed in Djibouti [53]. Isolates of *M. canetti* are often associated to a specific geographical area, the Horn of Africa. This species causes very rare cases of TB and is so far only found in humans and the natural reservoir is still unknown.

M. canetti has smooth and glossy colony morphology at the first stage of isolation and loses the smoothness or reverts after subsequent sub-culturing. Differentiation using biochemical tests is difficult and time consuming. The most rapid differentiation of *M. canetti* from other MTC isolates is by PCR-restriction analysis of the *hsp65* gene and by RD12 analysis [54].

1.4.6 *Mycobacterium caprae*

M. caprae was described as the causative agent of TB in goats, but it has been found in other animals too, such as cattle, deer and wild boar [55]. Some studies have even reported isolates from humans [56, 57]. For a long time this species was considered as *M. bovis*, because the biochemical tests results were similar to *M. bovis* and *M. bovis* BCG. Later on *M. caprae* has been differentiated from *M. bovis* because of the different sequence in the *gyrB* gene and susceptibility to PZA [55]. In contrast to the other MTC members *M. caprae* harbours most of the deletions such as RD9, RD7, RD8, RD10, RD5, RD6, RD12 and RD13 [55].

1.4.7 *Mycobacterium pinnipedii*

M. pinnipedii was isolated from Australian fur seal in May 1990 and March 1991, inhabiting the southern Australian coastal waters [58]. Similar organisms were subsequently recovered from the same animal species from Argentina, Uruguay, Great Britain and New Zealand and from a Brazilian tapir. So far there is only one case report from humans, a seal trainer with captive sea lions in Australia [59]. The growth of *M. pinnipedii* can be enhanced by media that contains sodium pyruvate. *M. pinnipedii* are closely related to *M. bovis*, but they differ in their loose cord formation, lack of the MPB70 antigen which is detectable in *M. bovis* and they are susceptible to PZA [58]. As *M. bovis* they have deletions in RD3, RD7, RD8, RD9 and RD10 regions, but have intact RD4, RD5 and RD6 regions. The *M. pinnipedii* isolates can be easily distinguished from the rest of the MTC by regional deletion analysis of PiD1 and PiD2 loci [59].

1.5 CHARACTERIZATION OF THE MTC

1.5.1 Phenotypic characterization

Accurate species identification of the MTC members is necessary in order to differentiate between human and zoonotic TB and to trace the source of infections. The traditional method of species identification is relying on the phenotypic character, which is based on biochemical testing including growth characteristics on different media and colony morphology. The colony morphology varies among the species of MTC ranging from flat smooth, domed glossy colonies to dry and rough colonies. Biochemical tests are dependent on the cultured isolates. For differentiation of the MTC various tests such as nitrate reduction, detection of niacin, growth in the presence of TCH, and catalase activity [60] may be used. All these tests are simple and inexpensive to perform but need an experienced person to interpret the results. The drawback of the tests is also that they take a long time, and do not differentiate very clearly between species of the MTC [61, 62]. They can also give different results between laboratories if they do not use the same protocol.

1.5.2 Genotypic characterization

The genotyping is based on the analysis of chromosomal DNA of MTC isolates. Genotyping methods are used for species identification as well as fingerprinting of individual isolates. The genotyping methods are very rapid and accurate compared to the phenotyping methods but they are expensive.

The development of DNA fingerprinting techniques for typing of MTC isolates during the last decade has led to an increasing number of studies of the molecular epidemiology of TB. Molecular typing techniques allow investigators to determine whether the strain causing disease in one patient is identical to that causing disease in another patient, in order to demonstrate whether transmission has taken place or not.

1.5.2.1 Spoligotyping

Spoligotyping is a PCR based assay that detects variability in the direct repeat region in the DNA of MTC. This region is present in all MTC strains in a unique locus which contains well conserved 36 bp repeats interspersed with non-repetitive short spacer sequences of 34 to 41 bp. The number of direct repeats per strain and thereby the presence of particular spacer sequences differs significantly between strains. The first generation spoligotyping that is used worldwide contains 43 spacers and is commercially available as a kit (Isogen Bioscience BV). Different *M. tuberculosis* strains have various combinations of the 43 spacers and these differences form the basis of the assay [51].

To visualize the DNA polymorphism in the direct repeat region of MTC strains the spacer sequences are amplified by PCR using biotin-labelled primers. The PCR products are then denatured and hybridized perpendicular to 43 oligonucleotides, which are covalently bound to a spoligo membrane [51]. Spoligotyping can be directly applied to detect and type the MTC bacteria in clinical samples. It is a good technique to differentiate and type the species of MTC and at the same time identifies different phylogenetic lineages of strains of MTC e.g, *M. bovis*, *M. africanum* and the Beijing genotype. In addition it can be applied to samples which have been stored for many years like paraffin-embedded samples and archaeological materials such as mummified bodies [5, 63, 64].

The advantages of spoligotyping are that it is:

- very simple
- rapid
- inexpensive to run
- does not need any sophisticated software
- does not need large amount of extracted and purified DNA
- can be done on nonviable bacteria

The results of the spoligotyping can be converted to octal values, compared to the international spoligotyping database SpolDB4 [65] and assigned to the phylogenetic lineages. The SpolDB4 contains more than 39,000 spoligopatterns collected from 122 different countries.

1.5.2.2 Restriction Fragment Length Polymorphism (RFLP)

The most widely applied and standardized molecular typing method for differentiation of MTC isolates is restriction fragment length polymorphism (RFLP), which is based on the detection of the insertion sequence *IS6110*. The *IS6110* element is specific to the MTC and found in almost all isolates of the MTC except in a few geographical variants. When it is found in the MTC the number and position of the *IS6110* element in the chromosomal DNA varies from one isolates to another. Thus the RFLP method is based on the differences in the *IS6110* copy numbers per strain ranging from 0 to about 25, and variability in the chromosomal positions of these

IS6110 insertion sequences [66]. The numbers and positions can be visualized and compared using internationally standardized protocols.

In 1993, van Embden and colleagues proposed a standardized method for performing IS6110 RFLP typing. In short DNA is extracted and restricted by the enzyme *PvuII*. The digested DNA is separated by gel electrophoresis and the DNA fragments are transferred to a nylon membrane. To visualize the fragments it is probed with a 245 bp sequence of IS6110 and detected using a chemiluminescence kit. The images are captured on x-ray films and then scanned and digitalized for computer analysis by the BioNumerics software (Applied Maths, Belgium). For MTC isolates with <5 IS6110 copies RFLP typing based on IS6110 is insufficiently discriminative [51] and it is therefore recommended to subtype them with another technique such as spoligotyping, polymorphic GC-rich sequence (PGRS) or Variable Number Tandem Repeat (VNTR) typing.

The IS6110 RFLP method is used:

- To verify or rule out suspected transmission
- To detect unsuspected transmissions and outbreaks of TB [67]
- To monitor transmission of strains with a specific drug resistance [22, 68-70]
- To distinguish endogenous reactivation from exogenous reinfection [71]
- To detect laboratory cross contamination and to identify individual strains of MTC [72, 73]
- To find out the emergence of specific strains or families of the strains in different regions of the world [74]
- To quantify the extent of recent transmission in a population [75]

A disadvantage of IS6110 RFLP typing is that it requires much more bacteria to yield sufficient DNA for analysis than PCR-based tests do. Therefore the genotyping laboratories are compelled to extended culture incubation periods to obtain sufficient quantities of DNA. As a result of this it can delay reporting of the results. Another disadvantage is that it provides insufficient discrimination between isolates with no or only low number of IS6110 copies.

1.5.2.3 Variable Number Tandem Repeat

VNTR typing is based on analysis of DNA segments containing tandem repeated sequences. The number of copies of the repeated sequence varies among different strains of MTC. The VNTR technique for genotyping is not only used for MTC but also for genotyping of other bacterial species such as *Bacillus anthracis*, and *Yersinia pestis* [76-78]. The VNTR genotyping technique is based on PCR amplification using primers specific for the flanking regions of each VNTR region and determination of the sizes of the amplicons. If non-labelled primers are used the size of the amplicons can be determined by simple gel electrophoresis. For high output analysis fluorescence labelled primers are used and the amplicons are analyzed using automated capillaries such as ABI 3700. It gives data in a simple and certain format based on the number of repetitive sequences in so called polymorphic micro or minisatellite regions [79].

The most used version of VNTR of mycobacterial interspersed repetitive units (MIRU) (tandem repeats of 40-100 bp) was based on analysis of 12 loci. The relative discriminatory power of VNTR and RFLP typing may vary, depending on the strain collection. A Study of 12 and 15 loci selected for genotyping of MTC has shown less discrimination when compared to the IS6110 RFLP genotyping for isolates with a high copy number of IS6110 insertions e.g., for the *M. tuberculosis* Beijing family [80, 81]. On the other hand MIRU-VNTR typing has better discriminating power than IS6110 RFLP for the isolates that contain low copy numbers of IS6110. To increase the discriminating power it is suggested to run a set of 24 MIRU-VNTR loci, which was proposed and standardized by Supply [82, 83].

Advantages of MIRU-VNTR typing over IS6110 RFLP are:

- As it is PCR based it can be performed on mycobacterial colonies without extensive DNA extractions
- It can be applied to various biological materials including nonviable material
- The results are expressed in simple numerical format, making it easy for exchange of data between different laboratories for comparisons and establishment of a central database.

1.6 LINEAGES OF THE MTC

According to the spolDB4 database [65] the MTC is classified into 62 genetic lineages, where *M. canetti*, *M. caprae* and *M. microti* all have one lineage each; *M. pinnipedii* two lineages; *M. bovis* three lineages; *M. africanum* four lineages and *M. tuberculosis* the most diversified has fifty lineages [29].

Most of the *M. tuberculosis* lineages reflect regions, countries, cities or places of high prevalence. For example the Latin American Mediterranean (LAM) lineage is dominating in South America, the Central Asian Strain (CAS) lineage is dominating in the Middle-East, Central Asia and South-Asia [84] while the East African Indian (EAI) lineage is more prevalent in South-East Asia [85]. Examples of lineages associated with the name of the countries are EAI1-SOM (reflecting Somalia), EAI-ETH (Ethiopia), EAI4_VNM (Vietnam), EAI8_MDG (Madagascar), EAI3-IND (India), EAI6_BGD (Bangladesh), LAM-Cam (Cameroon) and LAM-ZWE (Zimbabwe). Other lineages have cities or places included in their genotype [29]. There are also lineages designated by letters, like S, T, U and X. The T lineage is a common rather ill defined lineage [29] found in all different regions of the world. Certain of these lineages reflect areas where they have been found; T1_RUS (Russia), T2-Uganda (Uganda), T3_Eth (Ethiopia), T4-CE1 (Central-Europe), T3-Osaka and T5-Madrid. The T lineage includes high as well as low banding patterns of the IS6110 element [67]. On the contrary, the X lineage is well characterized by IS6110-RFLP as a low-banding family [86]. The zero lineages represent the Vietnamese genotype family characterized by the absence of IS6110 elements [29, 87].

1.7 DRUG RESISTANT TUBERCULOSIS

Wild type isolates of *M. tuberculosis* that have never been exposed to any anti-TB drugs almost never show any resistance [88]. DRTB develops from inadequate treatment of active pulmonary TB. Most of the time, it is caused by

- inconsistent or partial treatment,
- patients who do not take all their medicines regularly for the required period
- wrong treatment regimens prescribed
- poor quality of drugs

When the organisms become resistant after treatment within the same host it is called acquired resistance and when a resistant organism is transmitted to a different host it is said to be primary resistance.

Strains are defined as MDR if they are resistant to at least INH and rifampicin (RIF), the two most important drugs used for the treatment of the disease. When MDR strains develop further resistance to any member of the quinolones and at least to one of the anti-TB injectable drugs (kanamycin, capreomycin or amikacin), they are said to be extensively drug resistant (XDR) [12].

The emergence of drug resistance in the treatment of TB has complicated its management. It is thought that in countries confronted with the HIV epidemic, the overlap of TB has led to a rapid acceleration of active TB and to the emergence of MDRTB [89]. In the developing countries, shortage of resources hinder regular drug resistance analysis, hence the magnitude of this problem remains largely unidentified.

In several countries HIV has been associated with outbreaks of TB involving MDR strains which respond poorly to standard therapy [90]. Three countries, China, India and Russia account for 62% of the estimated global burden of MDRTB [88]. In addition to this burden XDRTB has raised interest and concern among clinicians and public health authorities around the world. In 2008, 45 countries had traced and reported XDRTB. Most concerning is that the proportion of XDRTB is very high in some countries, in Russia 10% of all the MDR cases are XDR, in Estonia 24%, in Donetsk Oblast of Ukraine 15%, in Azerbaijan 12.8%, and Japan has reported 30% XDR among MDR cases [12].

2 THE PRESENT INVESTIGATION

2.1 THE AIM OF THE FIRST STUDY

The aim of the study was to investigate the spread of DRTB in Swedish patients from the year 1994 to 2005 by molecular fingerprinting.

2.1.1 Specific purpose

To investigate the strain diversity and transmission pattern of MTC isolates from Sweden and to assign them to phylogentic lineages using spoligotyping.

2.2 THE AIM OF THE SECOND STUDY

The aim of the study was to investigate the presence and transmission of *M. tuberculosis* Beijing genotype in Sweden in relation to drug resistance from the year 1994 to 2008.

2.2.1 Specific purpose

To differentiate the Beijing genotype strains to different sublineages using different molecular markers.

2.3 MATERIALS AND METHODS

2.3.1 Materials for Study I

To perform a population based study of drug resistant TB, 400 isolates of MTC were obtained from all TB laboratories of Sweden during the years 1994-2005. These isolates were resistant to at least one of the first-line drugs. The five different TB laboratories were located at University hospitals of Sweden (Gothenburg, Linköping, Malmö /Lund, Stockholm and Umeå).

2.3.2 Materials for Study II

This was an extrapolation of the first study and followed up DRTB in Sweden from the years 1994-2008. In this study we analyzed 536 isolates recovered from 535 TB patients with special emphasis on the 70 *M. tuberculosis* isolates of Beijing genotype.

2.3.3 Methods used for the two studies

All isolates were tested by acid fast staining to confirm that they belonged to the mycobacterium genus. Identification for MTC was performed using the AccuProbe test (GenProbe Inc., San Diego, CA)

2.3.3.1 Susceptibility tests

All isolates of MTC were tested for drug susceptibility with standard methods using the Bactec 460 radiometry assay. Strains resistant to at least one of the first line drugs INH, RIF and EB were included in the study. During the major part of the study most isolates were also tested for susceptibility to SM. For the isolates obtained during the later years the susceptibility testing was performed using the BACTEC MIGIT960 (Becton Dickinson, Sparks, MD). All five Swedish TB laboratories had taken part in the external quality assurance program for drug susceptibility testing of *M. tuberculosis* provided by the Swedish TB reference laboratory at the Swedish Institute for Infectious Disease Control (SMI).

2.3.3.2 Genetic analysis

The isolates were cultured on Löwenstein-Jensen medium. Prior to the extraction of the DNA, the isolates were heat killed at 80°C for 20 minutes, followed by lysis using lysozyme, sodium chloride, sodium dodecyl sulphate, proteinase K and cetyl trimethyl ammonium bromide. DNA was extracted using the chloroform-isoamyl alcohol mixture and separation of the DNA content by using a micro centrifuge and by cold isopropanol [67]. DNA was quantified spectrophotometrically.

2.3.3.3 *Spoligotyping*

Following the standard protocols from the manufacturer (Isogen Bioscience BV, Maarsse, The Netherlands) spoligotyping was performed with the DRa biotinylated and DRb primers. The biotin-labeled (amplified) PCR product were denatured and hybridized to the probes which were covalently bound to a membrane using a miniblotted, and detected by a chemiluminescence kit [33]. The results were converted to octal numbers and compared to the international spoligotyping database SpolDB4 (<http://www.pasteur-guadeloupe.fr:8081/SITVITDemo>) and assigned to the phylogenetic lineages. The results were compared by visual examination and entered into the BioNumerics software (version 5.10) as character types. The individual spoligotypes were given their Spoligo-International-Type (SIT) number in accordance to their signatures found in the SpolDB4 database and assigned to the major phylogenetic lineages.

2.3.3.4 *IS6110-RFLP*

IS6110-RFLP was performed in accordance with the standard protocol described previously [91]. About 4.5µg chromosomal DNA was digested with the restriction enzyme *PvuII*, the fragments were separated by gel electrophoresis, and transferred to a hybrid positively charged membrane by Southern blotting. The membrane was hybridized using the probe *IS6110* labelled with horseradish peroxidase and detected using chemiluminescence kit. The autoradiography of RFLP was scanned and analyzed using the BioNumerics software (version 5.10) (Applied Maths, Kortrijk, Belgium). The *IS6110* RFLP patterns were analyzed as fingerprint types and the similarities were computed using the un-weighted pair group method of arithmetic average (UPGMA) and the Jaccard index. Clusters were defined when two or more MTC isolates from different patients showed 100% identical RFLP patterns.

2.3.3.5 *IS1547-RFLP*

IS1547-RFLP was carried out by Southern hybridization using the same blots (*PvuII*-digested DNA) as for *IS6110*-RFLP. The *IS1547* probe for hybridization was obtained by amplification using the primers 15F (5'-TGTGTGTGCCGCGAGGTGGG-3'), and 15R (5'-GCAATAGCTCCTATGGCAAGCGGC-3') [92, 93].

2.3.3.6 *RD polymorphism*

Genomic deletions of RD105, RD142, RD150, and RD181 were probed by PCR using primer sets described by Tsolaki [94]. The PCR products were analysed by agarose gel electrophoresis.

2.3.3.7 *Rv3135 gene*

Rv3135 gene analysis was carried out using the primers *Rv3135*-F (5'-TCGACTGC-CATAACCTG-3') and *Rv3135*-R (5'-GTGCTGGTCGAGAAGTGAATG-3') located 210 bp upstream from the *Rv3135* start site and 23 bp downstream from the

stop codon, respectively [95]. The variations of PCR products were detected by gel electrophoresis.

2.3.3.8 *mutT2 and mutT4 gene polymorphism*

mutT2 and *mutT4* gene polymorphism was performed by sequencing of these genes. The DNAs were amplified using the primers mutT2-F (5'-TCCGGATGATGATTTACCTCC-3') and mutT2-R (5'-TCCGCCGGGTCGGGGAC-3') and mutT4-F (5'-TCGAAGGTGGGCAAATCGTG-3') and mutT4-R (5'-TGGGGTTCGCTGGAA-GTGG-3'). The PCR products were purified using spin columns supplied by GE Healthcare. The mutations GGA to CGA (Gly to Arg) at codon position 58 for mutT2 and CGG to GGG (Arg to Gly) at codon position 48 for mutT4 were detected using the ABI Big Dye v.3.1 sequencing kit [96, 97].

2.4 RESULTS AND DISCUSSION

2.4.1 Study I

According to the drug susceptibility patterns 271 isolates were resistant to only one drug and 129 isolates were resistant to two or more drugs of which 46 were MDR and one was XDR.

All isolates were characterized by spoligotyping. The major phylogenetic lineages were Central Asian Strain (CAS), East-African-Indian (EAI), Haarlem (H) Latin-American and Mediterranean (LAM), "Manu" family, X lineage, S lineage and the ill-defined T lineage. Out of 400 isolates, 327 (82%) were clustered into 38 spoligo-clusters and in total we detected 111 different spoligotype patterns.. The size of the spoligo-cluster varied from 2-109 isolates per cluster. The remaining 73 (18%) spoligopatterns were unique. Of 111 different spoligotype patterns 84 were already described in the international database SpolDB4. The remaining 27 spoligotype patterns were not found in the SpolDB4. The most dominant lineage in this study was the T lineage (n=174), which contained all members of the cluster SMI-049. Other major genotypes were the Beijing genotype (n=48), EAI lineage (n=36), H (n=36), LAM (n=32), CAS (n=19), and KILI family (n=12). Some minor lineages like the U family (n=9), S family (n=4) and X family (n=2) were also detected. Though a majority (n= 178) of the foreign-born patients come from the Horn of Africa we found only one isolate of the AFRI3 family.

The distribution of the global strain lineages in relation to the geographical origin of the patients is depicted in Table 1. The foreign-born patients came from several regions of the world with dominance of Africa (n=213) followed by the Middle East and Central Asia (n=55), South East Asia (n= 41), Europe (n=14) and South America (n=12). The lineages of isolates of foreign-born patients reflected to a great extent their country of origin.

Table 1 Global strain lineages of isolates in relation to the origin of patients (number and region or country of origin of patients with DRTB)

Lineage	Sweden	Africa	South East Asia	Middle East central Asia	Europe	South America	Unknown	Total	Clusters	Patients in cluster
T1	13	15	2	9	9		1	49	7	23
T2*	13	87		3	3	3		109	3	103
T3		14			2			16	3	12
Beijing	5	6	20	17				48	6	16
EAI	2	21	13					36	3	12
Haarlem	5	13	3	10	3	2		36	5	16
LAM	7	12		4	3	5	1	32	4	10
CAS		24		7				31	2	7
U	2	2	1	3	1			9	2	4
S		1			2	1		4		
X		2						2		
AFRI3		1						1		
Undefined	6	15	2	2		1	1	27		
Total	53	213	41	55	23	12	3	400	35	203

East-African Indian (EAI): EAI1Som; EAI1IND; EAI2Manilla; EAI3; EAI4; EAI5; EAI6DGI; EAI8MDG. Haarlem (H): H1-H4. Central Asian Strain (CAS): CAS undefined; CAS1KILI; CAS1Delhi. Latin-American-Mediterranean (LAM): LAM1; LAM3-7; LAM9; LAM10; LAM11-ZWE.

*Including all 96 patients in cluster SMI-049

All isolates that were identical by RFLP and contained more than five bands of *IS6110* were also identical by spoligotyping. Using the *IS6110* as a molecular marker we observed 228 different RFLP patterns. By combining the RFLP and spoligotyping pattern, 203 (52%) isolates clustered into 35 different clusters. The size of the clusters varied from 2-96. Of the 203 patients with clustered isolates, 178 (88%) were foreign-born and 25 (12%) Swedish-born. The majority of the clusters comprised only 2-4 individuals per cluster but one exceptionally large INH resistant cluster (SMI-049), which comprised 96 isolates was detected. The cluster SMI-049 was first identified with two individuals per year from 1996 until 1998; but in 1999, 19 new patients were identified with isolates with this identical *IS6110* pattern. Because most reported patients from cluster SMI-049 in the beginning were from the Horn of Africa (Somalia), it was thought that the TB transmission probably had occurred prior to their arrival in Sweden. Strains of this cluster then spread gradually to different foreign-born ethnic groups and to Swedish-born in the later phase. In 2005, there was an outbreak at a Swedish day-care centre caused by a patient who had a strain belonging to cluster SMI-049. Up to date (March 2010), 114 patients have by culture been verified to belong to cluster SMI-049, 21 being Swedish-born. The findings of Swedish-born patients during the later phase of the outbreak indicate that the transmission has

gradually taken hold in the Swedish-born population. The cluster SMI-049 has circulated in the Swedish society for 13 years, yet there was no change in banding patterns of IS6110-RFLP, except for one patient.

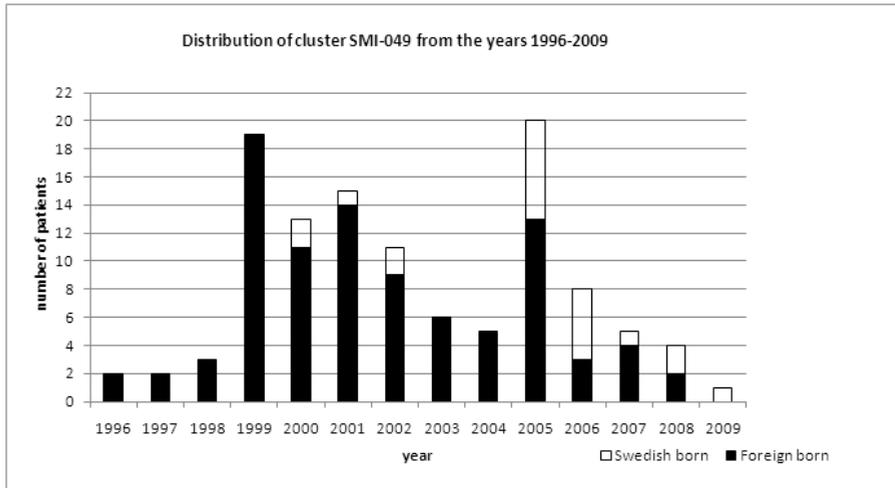


Figure 1. Distribution of cluster SMI-049 during the years 1996-2009.

So far, this outbreak of DRTB is one of the largest clusters ever reported, and was an important warning signal to the Swedish health authorities. Application of the molecular techniques and specific markers allowed us to monitor the spread of resistant TB isolates in Sweden.

2.4.2 Study II

Using spoligotyping we identified 70 DR isolates of the Beijing genotype. The majority of these isolates were mono-resistant, 17 strains were MDR and one strain was defined as XDR. Most patients with Beijing strains were foreign-born, coming from countries where the Beijing lineage is highly prevalent. We analysed the 70 DR Beijing isolates by IS6110 and IS1547 RFLP for the same Southern blotted membranes, spoligotyping, Rv1335 gene polymorphism, regional deletions RD105, RD142, RD150, RD181 and the mutations for *mut2*, *mut4* genes. We identified 10 clusters using the IS6110 RFLP. The major cluster consisted of five patients and four of them came from the former Soviet Union. Other clusters consisted of two to three patients and most of the patients in a cluster originated from the same country. Only two out of the six Swedish-born patients were included in two different clusters whereas the other four had unique RFLP patterns. Contact tracing confirmed the linkage for one Swedish-born patient with a patient from Bhutan. Both of them had participated in the same indoor recreational activity during the wintertime of 2002. Otherwise, there were no large clusters of Beijing isolates and there were no reports on outbreaks caused by Beijing strains. Thus so far no extensive spread of DR Beijing strains has taken place in Sweden.

MDR was significantly higher in Beijing strains 17/69 (24%) than MDR in non-Beijing strains 59/466 (13%) [p value=0.0138, 95% CI= (0.0137; 0.2258)]. Forty-two of the 63 foreign-born TB patients infected with DR Beijing isolates were females. Thus the number of females was higher than males in the group of patients with Beijing isolates, which is unusual but in accordance with the findings in a study from Malawi [98]. In order to understand the reasons for this difference further investigation is needed. The majority of the patients (52%) were in the age group of 25-44 years and 21% in the age group 15-24 years. Using different genotyping methods the 70 isolates were allocated to 11 different genetic sublineages. The majority of the isolates (54/70) belonged to the typical “modern” Beijing having a 1.02-kb fragment of the *Rv3135* gene and 15/70 were defined as atypical Beijing containing a 1.97-kb fragment of the *Rv3135* gene. All the atypical Beijing isolates had a mutation (Gly to Arg) at codon position 58 of the *mufT2* gene. One strain, BTB 04-003, lacked the deletion of RD105 which is considered to be the marker for Beijing strains, and had a 0.150-kb fragment in *Rv3135* gene rather than 1.97-kb and 1.02-kb bands. This strain may be either an early ancestral variant occurring before the RD105 deletion, or may have developed independently through selective pressure. Thus, even though countries with a high prevalence of DR Beijing strains are not far from Sweden, still there is little indication of spread of the DR Beijing genotype in Sweden.

Table 2. Polymorphisms of *M. tuberculosis* isolates of Beijing genotype.

Genotypic sublineage	Number of isolates(n=70)	SIT ^c	Region of difference (RD) ^a				Rv3135 ^d	Mutation in <i>mufT</i> genes ^b		
			105	181	150	142		<i>mufT2</i>	<i>mufT4</i>	IS1547 ^e
1	1	265	+	+	+	+	0.15kb	-	-	8
2	13	1	-	-	+	+	at	-	+	5
3	1	1	-	-	+	+	at	-	+	6
4	1	1	-	-	+	+	at	-	+	7
5	41	1	-	-	+	+	t	+	+	1
6	3	1	-	-	+	+	t	+	+	2
7	1	1	-	-	+	+	t	+	+	3
8	3	265	-	-	+	+	t	+	+	1
9	1	1	-	-	+	-	t	+	+	1
10	4	1	-	-	-	+	t	+	+	1
11	1	1	-	-	-	+	t	+	+	4

^a presence (+) or absence (-) of the specific genomic region

^b presence (+) or absence (-) of the specific mutation

^c spoligotype international type

^d sublineage 1: a 0.15-kb PCR product, sublineage 2-4: "atypical" isolates, with a 1.97-kb PCR product, sublineage 5-11: "typical" isolates, with a 1.02-kb PCR product

^e pattern 1: three bands (1.7, 2.1, and 2.5 kb), pattern 2: two bands (2.1 and 2.5kb), pattern 3-7: individual patterns, all containing the 2.5 kb band, pattern 8: a single 2.5 kb band absent

2.5 CONCLUSIONS AND REMARKS

This molecular characterization of MTC isolates from Sweden is the first population based study of DRTB. Routine molecular analysis of DR MTC strains identified suspected and unsuspected chains of transmission of TB. Additionally, for some TB patients, the typing could discriminate between re-infection and relapse from a previous episode. The study revealed the extensive spread of an INH resistant TB strain in Sweden.

The study also demonstrated the importance of molecular typing of DRTB strains as a powerful tool to monitor and identify deficiencies in national TB control programs. We recommend analyzing all isolates of MTC from Swedish patients by molecular fingerprinting to identify sources of infection, to follow known outbreaks as well as to identify unknown outbreaks at early stages and intensify the treatment of infected persons. Screening of asylum seekers and immigrants that come from high-incidence countries would help to avoid and stop transmission of TB from foreign-born patients.

Further studies and analyses might be needed to answer questions, such as why the strain of cluster SMI-049 spread rapidly within specific ethnic groups, whether this outbreak strain is more virulent than other strains, and why the Beijing genotype does not take hold in Sweden.

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4 REFERENCES

1. Zink, A., et al., *Molecular analysis of skeletal tuberculosis in an ancient Egyptian population*. J Med Microbiol, 2001. **50**(4): p. 355-66.
2. Donald, P.R. and P.D. van Helden, *The global burden of tuberculosis--combating drug resistance in difficult times*. N Engl J Med, 2009. **360**(23): p. 2393-5.
3. De Backer, A.I., et al., *Tuberculosis: epidemiology, manifestations, and the value of medical imaging in diagnosis*. Jbr-Btr, 2006. **89**(5): p. 243-50.
4. Vashishtha, V.M., *WHO Global Tuberculosis Control Report 2009: Tuberculosis elimination is a distant dream*. Indian Pediatr, 2009. **46**(5): p. 401-2.
5. Donoghue, H.D., et al., *Tuberculosis: from prehistory to Robert Koch, as revealed by ancient DNA*. Lancet Infect Dis, 2004. **4**(9): p. 584-92.
6. Arriaza, B.T., et al., *Pre-Columbian tuberculosis in northern Chile: molecular and skeletal evidence*. Am J Phys Anthropol, 1995. **98**(1): p. 37-45.
7. Hershkovitz, I., et al., *Detection and molecular characterization of 9,000-year-old Mycobacterium tuberculosis from a Neolithic settlement in the Eastern Mediterranean*. PLoS One, 2008. **3**(10): p. e3426.
8. Smith, I., *Mycobacterium tuberculosis pathogenesis and molecular determinants of virulence*. Clin Microbiol Rev, 2003. **16**(3): p. 463-96.
9. Kaufmann, S.H. and U.E. Schaible, *100th anniversary of Robert Koch's Nobel Prize for the discovery of the tubercle bacillus*. Trends Microbiol, 2005. **13**(10): p. 469-75.
10. Grange, J.M. and A. Zumla, *The global emergency of tuberculosis: what is the cause?* J R Soc Promot Health, 2002. **122**(2): p. 78-81.
11. Zumla, A., et al., *Reflections on the white plague*. Lancet Infect Dis, 2009. **9**(3): p. 197-202.
12. Sheno, S. and G. Friedland, *Extensively drug-resistant tuberculosis: a new face to an old pathogen*. Annu Rev Med, 2009. **60**: p. 307-20.
13. Dye, C., et al., *Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project*. Jama, 1999. **282**(7): p. 677-86.
14. *Trends in tuberculosis--United States, 2007*. MMWR Morb Mortal Wkly Rep, 2008. **57**(11): p. 281-5.
15. Puranen, B., *TUBERCULOSIS "The Occurrence and Causes in Sweden 1750-2000"*. 2003. p. Chapter 9.
16. Romanus, V., *The Swedish Tuberculosis Index 2001 and 2002*, in *Swedish Institute for Infectious Disease Control and Swedish Heart Lung Foundation*. 2004. p. 21.
17. Lundquist, J., *BCG vaccination in Sweden*. Pediatrics, 1949. **4**(4): p. 430.
18. Sjogren, I. and I. Sutherland, *The risk of tuberculous infection in Sweden*. Tubercle, 1975. **56**(2): p. 97-112.
19. Szwedzyk, R., et al., *Molecular epidemiological studies of Mycobacterium bovis infections in humans and animals in Sweden*. J Clin Microbiol, 1995. **33**(12): p. 3183-5.
20. Jonsson, J. and F. Hansdotter. *Statistik för tuberkulos*. 2009 2009-09-21 Available from: <http://www.smi.se/statistik/tuberkulos>.
21. Romanus, V., *Selective BCG vaccination in a country with low incidence of tuberculosis*. Euro Surveill, 2006. **11**(3): p. 14-7.
22. Ghebremichael, S., et al., *Molecular epidemiology of drug-resistant tuberculosis in Sweden*. Microbes Infect, 2008. **10**(6): p. 699-705.
23. Evans, J.T., et al., *Cluster of human tuberculosis caused by Mycobacterium bovis: evidence for person-to-person transmission in the UK*. Lancet, 2007. **369**(9569): p. 1270-6.
24. Lewerin, S.S., et al., *Outbreak of Mycobacterium tuberculosis infection among captive Asian elephants in a Swedish zoo*. Vet Rec, 2005. **156**(6): p. 171-5.

25. Brosch, R., et al., *A new evolutionary scenario for the Mycobacterium tuberculosis complex*. Proc Natl Acad Sci U S A, 2002. **99**(6): p. 3684-9.
26. Huard, R.C., et al., *PCR-based method to differentiate the subspecies of the Mycobacterium tuberculosis complex on the basis of genomic deletions*. J Clin Microbiol, 2003. **41**(4): p. 1637-50.
27. Hoffner, S.E., et al., *Biochemical heterogeneity of Mycobacterium tuberculosis complex isolates in Guinea-Bissau*. J Clin Microbiol, 1993. **31**(8): p. 2215-7.
28. Niemann, S., E. Richter, and S. Rusch-Gerdes, *Biochemical and genetic evidence for the transfer of Mycobacterium tuberculosis subsp. caprae Aranaz et al. 1999 to the species Mycobacterium bovis Karlson and Lessel 1970 (approved lists 1980) as Mycobacterium bovis subsp. caprae comb. nov.* Int J Syst Evol Microbiol, 2002. **52**(Pt 2): p. 433-6.
29. Brudey, K., et al., *Mycobacterium tuberculosis complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology*. BMC Microbiol, 2006. **6**: p. 23.
30. Gagneux, S. and P.M. Small, *Global phylogeography of Mycobacterium tuberculosis and implications for tuberculosis product development*. Lancet Infect Dis, 2007. **7**(5): p. 328-37.
31. Collins, C.H. and J.M. Grange, *The bovine tubercle bacillus*. J Appl Bacteriol, 1983. **55**(1): p. 13-29.
32. Robinson, P., D. Morris, and R. Antic, *Mycobacterium bovis as an occupational hazard in abattoir workers*. Aust N Z J Med, 1988. **18**(5): p. 701-3.
33. Muller, B., et al., *African 1, an epidemiologically important clonal complex of Mycobacterium bovis dominant in Mali, Nigeria, Cameroon, and Chad*. J Bacteriol, 2009. **191**(6): p. 1951-60.
34. Ciesielski, S.D., *BCG vaccination and the PPD test: what the clinician needs to know*. J Fam Pract, 1995. **40**(1): p. 76-80.
35. Frank, W., et al., *Mycobacterium microti--pulmonary tuberculosis in an immunocompetent patient*. Wien Klin Wochenschr, 2009. **121**(7-8): p. 282-6.
36. Huitema, H. and F.H. Jaartsveld, *Mycobacterium microti infection in a cat and some pigs*. Antonie Van Leeuwenhoek, 1967. **33**(2): p. 209-12.
37. Taylor, C., et al., *Mycobacterium microti isolated from two pigs*. Vet Rec, 2006. **159**(2): p. 59-60.
38. Jahans, K., et al., *Isolation of Mycobacterium microti from a male Charolais-Hereford cross*. Vet Rec, 2004. **155**(12): p. 373-4.
39. Deforges, L., et al., *First isolation of Mycobacterium microti (Llama-type) from a dog*. Vet Microbiol, 2004. **103**(3-4): p. 249-53.
40. Niemann, S., et al., *Two cases of Mycobacterium microti derived tuberculosis in HIV-negative immunocompetent patients*. Emerg Infect Dis, 2000. **6**(5): p. 539-42.
41. van Soolingen, D., et al., *Diagnosis of Mycobacterium microti infections among humans by using novel genetic markers*. J Clin Microbiol, 1998. **36**(7): p. 1840-5.
42. Manabe, Y.C., C.P. Scott, and W.R. Bishai, *Naturally attenuated, orally administered Mycobacterium microti as a tuberculosis vaccine is better than subcutaneous Mycobacterium bovis BCG*. Infect Immun, 2002. **70**(3): p. 1566-70.
43. Frota, C.C., et al., *Genome structure in the vole bacillus, Mycobacterium microti, a member of the Mycobacterium tuberculosis complex with a low virulence for humans*. Microbiology, 2004. **150**(Pt 5): p. 1519-27.
44. Lutze-Wallace, C., et al., *Isolation of a Mycobacterium microti-like organism from a rock hyrax (Procapra capensis) in a Canadian zoo*. Canadian Veterinary Journal-Revue Veterinaire Canadienne, 2006. **47**(10): p. 1011-3.
45. Castets, M., et al., *[Tuberculosis bacilli of the African type: preliminary note]*. Rev Tuberc Pneumol (Paris), 1968. **32**(2): p. 179-84.
46. Kallenius, G., et al., *Evolution and clonal traits of Mycobacterium tuberculosis complex in Guinea-Bissau*. J Clin Microbiol, 1999. **37**(12): p. 3872-8.

47. Viana-Niero, C., et al., *Genetic diversity of Mycobacterium africanum clinical isolates based on IS6110-restriction fragment length polymorphism analysis, spoligotyping, and variable number of tandem DNA repeats*. J Clin Microbiol, 2001. **39**(1): p. 57-65.
48. Asiiimwe, B.B., et al., *Mycobacterium tuberculosis Uganda genotype is the predominant cause of TB in Kampala, Uganda*. Int J Tuberc Lung Dis, 2008. **12**(4): p. 386-91.
49. Brudey, K., et al., *Mycobacterium africanum genotyping using novel spacer oligonucleotides in the direct repeat locus*. J Clin Microbiol, 2004. **42**(11): p. 5053-7.
50. Goguet de la Salmoniere, Y.O., et al., *Evaluation of spoligotyping in a study of the transmission of Mycobacterium tuberculosis*. J Clin Microbiol, 1997. **35**(9): p. 2210-4.
51. Kamerbeek, J., et al., *Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and epidemiology*. J Clin Microbiol, 1997. **35**(4): p. 907-14.
52. Pfyffer, G.E., et al., *Mycobacterium canettii, the smooth variant of M. tuberculosis, isolated from a Swiss patient exposed in Africa*. Emerg Infect Dis, 1998. **4**(4): p. 631-4.
53. Miltgen, J., et al., *Two cases of pulmonary tuberculosis caused by Mycobacterium tuberculosis subsp canetti*. Emerg Infect Dis, 2002. **8**(11): p. 1350-2.
54. Goh, K.S., et al., *Rapid differentiation of "Mycobacterium canettii" from other Mycobacterium tuberculosis complex organisms by PCR-restriction analysis of the hsp65 gene*. J Clin Microbiol, 2001. **39**(10): p. 3705-8.
55. Aranaz, A., et al., *Elevation of Mycobacterium tuberculosis subsp. caprae Aranaz et al. 1999 to species rank as Mycobacterium caprae comb. nov., sp. nov.* Int J Syst Evol Microbiol, 2003. **53**(Pt 6): p. 1785-9.
56. Kubica, T., S. Rusch-Gerdes, and S. Niemann, *Mycobacterium bovis subsp. caprae caused one-third of human M. bovis-associated tuberculosis cases reported in Germany between 1999 and 2001*. J Clin Microbiol, 2003. **41**(7): p. 3070-7.
57. Prodinger, W.M., et al., *Infection of red deer, cattle, and humans with Mycobacterium bovis subsp. caprae in western Austria*. J Clin Microbiol, 2002. **40**(6): p. 2270-2.
58. Cousins, D.V., et al., *Tuberculosis in seals caused by a novel member of the Mycobacterium tuberculosis complex: Mycobacterium pinnipedii sp. nov.* Int J Syst Evol Microbiol, 2003. **53**(Pt 5): p. 1305-14.
59. Bigi, F., et al., *Identification of genetic markers for Mycobacterium pinnipedii through genome analysis*. Fems Microbiology Letters, 2005. **248**(2): p. 147-52.
60. Frothingham, R., et al., *Phenotypic and genotypic characterization of Mycobacterium africanum isolates from West Africa*. J Clin Microbiol, 1999. **37**(6): p. 1921-6.
61. Djelouadji, Z., et al., *A Single-Step Sequencing Method for the Identification of Mycobacterium tuberculosis Complex Species*. PLoS Negl Trop Dis, 2008. **2**(6): p. e253.
62. Springer, B., et al., *Two-laboratory collaborative study on identification of mycobacteria: molecular versus phenotypic methods*. J Clin Microbiol, 1996. **34**(2): p. 296-303.
63. Qian, L., et al., *Retrospective analysis of the Beijing family of Mycobacterium tuberculosis in preserved lung tissues*. J Clin Microbiol, 1999. **37**(2): p. 471-4.
64. van der Zanden, A.G., et al., *Simultaneous detection and strain differentiation of Mycobacterium tuberculosis complex in paraffin wax embedded tissues and in stained microscopic preparations*. Mol Pathol, 1998. **51**(4): p. 209-14.
65. <http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/>
66. Van Soolingen, D., *Molecular epidemiology of tuberculosis and other mycobacterial infections: main methodologies and achievements*. J Intern Med, 2001. **249**(1): p. 1-26.
67. Hermans, P.W., et al., *Analysis of the population structure of Mycobacterium tuberculosis in Ethiopia, Tunisia, and The Netherlands: usefulness of DNA*

- typing for global tuberculosis epidemiology. *J Infect Dis*, 1995. **171**(6): p. 1504-13.
68. Frieden, T.R., et al., *The molecular epidemiology of tuberculosis in New York City: the importance of nosocomial transmission and laboratory error*. *Tuber Lung Dis*, 1996. **77**(5): p. 407-13.
 69. Frieden, T.R., et al., *A multi-institutional outbreak of highly drug-resistant tuberculosis: epidemiology and clinical outcomes*. *Jama*, 1996. **276**(15): p. 1229-35.
 70. Moro, M.L., et al., *An outbreak of multidrug-resistant tuberculosis involving HIV-infected patients of two hospitals in Milan, Italy*. *Italian Multidrug-Resistant Tuberculosis Outbreak Study Group*. *AIDS*, 1998. **12**(9): p. 1095-102.
 71. Sonnenberg, P., et al., *HIV-1 and recurrence, relapse, and reinfection of tuberculosis after cure: a cohort study in South African mineworkers*. *Lancet*, 2001. **358**(9294): p. 1687-93.
 72. Small, P.M., et al., *Molecular strain typing of Mycobacterium tuberculosis to confirm cross-contamination in the mycobacteriology laboratory and modification of procedures to minimize occurrence of false-positive cultures*. *J Clin Microbiol*, 1993. **31**(7): p. 1677-82.
 73. Bauer, J., et al., *False-positive results from cultures of Mycobacterium tuberculosis due to laboratory cross-contamination confirmed by restriction fragment length polymorphism*. *J Clin Microbiol*, 1997. **35**(4): p. 988-91.
 74. Mokrousov, I., *Genetic geography of Mycobacterium tuberculosis Beijing genotype: a multifacet mirror of human history?* *Infect Genet Evol*, 2008. **8**(6): p. 777-85.
 75. Murray, M.B., *Molecular epidemiology and the dynamics of tuberculosis transmission among foreign-born people*. *CMAJ*, 2002. **167**(4): p. 355-6.
 76. Keim, P., et al., *Molecular diversity in Bacillus anthracis*. *J Appl Microbiol*, 1999. **87**(2): p. 215-7.
 77. Le Fleche, P., et al., *A tandem repeats database for bacterial genomes: application to the genotyping of Yersinia pestis and Bacillus anthracis*. *BMC Microbiol*, 2001. **1**: p. 2.
 78. Supply, P., et al., *Variable human minisatellite-like regions in the Mycobacterium tuberculosis genome*. *Mol Microbiol*, 2000. **36**(3): p. 762-71.
 79. Mazars, E., et al., *High-resolution minisatellite-based typing as a portable approach to global analysis of Mycobacterium tuberculosis molecular epidemiology*. *Proc Natl Acad Sci U S A*, 2001. **98**(4): p. 1901-6.
 80. Allix-Beguec, C., et al., *Standardised PCR-based molecular epidemiology of tuberculosis*. *Eur Respir J*, 2008. **31**(5): p. 1077-84.
 81. Kremer, K., et al., *Use of variable-number tandem-repeat typing to differentiate Mycobacterium tuberculosis Beijing family isolates from Hong Kong and comparison with IS6110 restriction fragment length polymorphism typing and spoligotyping*. *J Clin Microbiol*, 2005. **43**(1): p. 314-20.
 82. Supply, P., et al., *Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of Mycobacterium tuberculosis*. *J Clin Microbiol*, 2006. **44**(12): p. 4498-510.
 83. Oelemann, M.C., et al., *Assessment of an optimized mycobacterial interspersed repetitive-unit-variable-number tandem-repeat typing system combined with spoligotyping for population-based molecular epidemiology studies of tuberculosis*. *J Clin Microbiol*, 2007. **45**(3): p. 691-7.
 84. Bhanu, N.V., et al., *Predominance of a novel Mycobacterium tuberculosis genotype in the Delhi region of India*. *Tuberculosis (Edinb)*, 2002. **82**(2-3): p. 105-12.
 85. Douglas, J.T., et al., *Characterization of the Manila family of Mycobacterium tuberculosis*. *J Clin Microbiol*, 2003. **41**(6): p. 2723-6.
 86. Warren, R.M., et al., *Clonal expansion of a globally disseminated lineage of Mycobacterium tuberculosis with low IS6110 copy numbers*. *J Clin Microbiol*, 2004. **42**(12): p. 5774-82.
 87. Soini, H., et al., *Transmission dynamics and molecular characterization of Mycobacterium tuberculosis isolates with low copy numbers of IS6110*. *J Clin Microbiol*, 2001. **39**(1): p. 217-21.

88. Aziz, M.A., et al., *Epidemiology of antituberculosis drug resistance (the Global Project on Anti-tuberculosis Drug Resistance Surveillance): an updated analysis*. Lancet, 2006. **368**(9553): p. 2142-54.
89. Lee, S.K., et al., *Multidrug-resistant tuberculosis*. Ann Acad Med Singapore, 1995. **24**(3): p. 442-6.
90. Corbett, E.L., et al., *The growing burden of tuberculosis: global trends and interactions with the HIV epidemic*. Arch Intern Med, 2003. **163**(9): p. 1009-21.
91. van Embden, J.D., et al., *Strain identification of Mycobacterium tuberculosis by DNA fingerprinting: recommendations for a standardized methodology*. J Clin Microbiol, 1993. **31**(2): p. 406-9.
92. Fang, Z. and K.J. Forbes, *A Mycobacterium tuberculosis IS6110 preferential locus (ipl) for insertion into the genome*. J Clin Microbiol, 1997. **35**(2): p. 479-81.
93. Mokrousov, I., et al., *Phylogenetic reconstruction within Mycobacterium tuberculosis Beijing genotype in northwestern Russia*. Res Microbiol, 2002. **153**(10): p. 629-37.
94. Tsolaki, A.G., et al., *Genomic deletions classify the Beijing/W strains as a distinct genetic lineage of Mycobacterium tuberculosis*. J Clin Microbiol, 2005. **43**(7): p. 3185-91.
95. Musser, J.M., A. Amin, and S. Ramaswamy, *Negligible genetic diversity of mycobacterium tuberculosis host immune system protein targets: evidence of limited selective pressure*. Genetics, 2000. **155**(1): p. 7-16.
96. Ebrahimi-Rad, M., et al., *Mutations in putative mutator genes of Mycobacterium tuberculosis strains of the W-Beijing family*. Emerg Infect Dis, 2003. **9**(7): p. 838-45.
97. Rindi, L., et al., *Evolutionary pathway of the Beijing lineage of Mycobacterium tuberculosis based on genomic deletions and mutT genes polymorphisms*. Infect Genet Evol, 2009. **9**(1): p. 48-53.
98. Glynn, J.R., et al., *Mycobacterium tuberculosis Beijing genotype, northern Malawi*. Emerg Infect Dis, 2005. **11**(1): p. 150-3.

