Tuberculosis– meeting the challenge of a global pandemic at molecular level

Thesis submitted as partial fulfilment for the PhD in medicine

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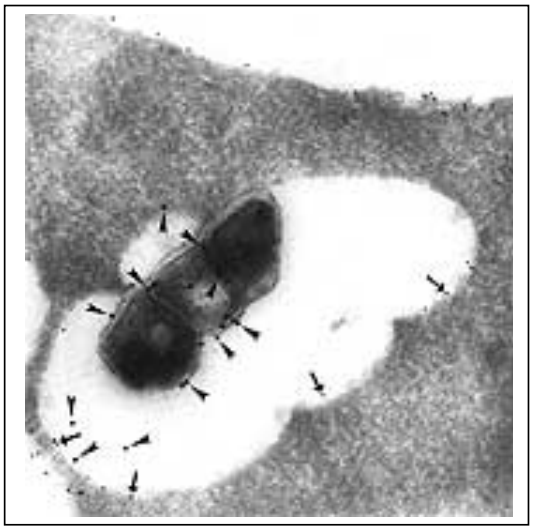

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Dedicated to Guro, Liv, Ola and Sigrid, my wife and my children.

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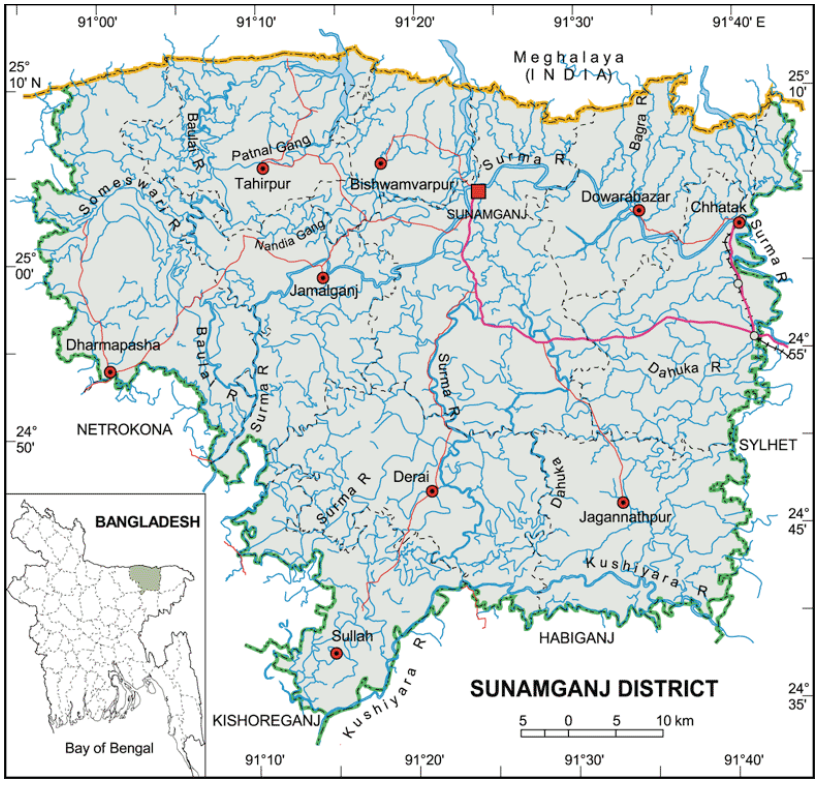
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ABBREVIATIONS

AFLP	amplified fragment-length polymorphism
BL	Beijing lineage
bp	base pair
BRAC	Bangladesh Rural Advancement Committee
CCS	critical community size
CD	cluster of differentiation
CDC	Centre for Disease Control (US Government, Atlanta)
CHW	community health worker
COPD	chronic obstructive pulmonary disease
DA	Destiny Associates
DNA	deoxyribonucleic acid
DOTS	directly observed treatment short-course
DR	direct repeat
EEA1	early-endosomal autoantigen 1
EMB or E	ethambutol
FLiP	fast ligation-mediated PCR
GoB	Government of Bangladesh
HCW	health care worker
HIV	human immunodeficiency virus
ICCDR,B	Centre for Health and Population Research, Bangladesh
IL	inter leukin
INF	Interferon
INH or I	Isoniazide
IS	inter section
IUATLD	International Union Against TB and Lung Disease
KP	Koch's phenomenon
LAM	Lipoarabinomannam
M. tuberculosis	*Mycobacterium tuberculosis*
ManLAM	mannosylated lipoarabinomannam
mce	mycobacterium cell entry operon
MCH	major histocompatibility complex
MDR-TB	multi drug resistant tuberculosis
MIRU-VNTR	MIRU variable-number tandem repeats
MMR	macrophage mannose receptor
MPT	secreted mycobacterial protein
MTC	*M. tuberculosis* complex
Mφ	Macrophage
NGO	non-government organisation
NIPH	Norwegian Institute of Public Health
NO	nitric oxide
NSDP	NGO service delivery program
NTM	non-tuberculous mycobacteriae
NTP	national TB program
OR	odds ratio
PAS	para-aminosalicylic acid
PCR	polymerase chain reaction

PE	proline-glutamic acid motif protein family
PGG	principal genetic group
PGRS	polymorphic repetitive sequences
PHC	primary health care
PMTR	polymorphic tandem repeats
PPE	proline-proline-glutamic acid motif protein family
PZA or P	Pyrazinamide
RD	regions of differences
RFLP	restriction fragment length polymorphism
RMP or R	Rifampicin
RNA	ribonucleic acid
RNI	reactive nitrogen intermediates
ROM	reactive oxygen intermediates
*rpo*B	B subunit of RNA polymerase gene
SM or S	Streptomycin
ss-	sputum-smear negative
ss+	sputum-smear positive
TB	Tuberculosis
TGF	transforming growth factor
Th	T helper cell
TLR	toll-like receptor
TNF	tumour necrosis factor
TS	traditional strains
TST	tuberculin skin test
UHC	Upazilla Health Complex
WHO	World Health Organisation

LIST OF PAPERS

This thesis is based on the following scientific papers assigned in the text by roman numerals:

I. Storla DG, Rahim Z, Islam MA, Plettner S, Begum V, Mannsaaker T, Myrvang B, Bjune G, Dahle UR. Heterogeneity of *Mycobacterium tuberculosis* isolates in Sunamganj District, Bangladesh. Scand J Infect Dis 2006;38 (8):593-6.

II. Storla DG, Rahim Z, Islam MA, Plettner S, Begum V, Myrvang B, Bjune G, Rønnild E, Dahle UR, Mannsåker T. Drug resistance of Mycobacterium tuberculosis in the Sunamganj District of Bangladesh. Scand J Infect Dis, 2007. 39(2): 142-5.

III. Storla, DG, Yimer S & Bjune G. A systematic review of delay in the diagnosis and treatment of tuberculosis. BMC Public Health, 2008. Jan 14;8:15.

IV. Storla, DG, Yimer S & Bjune G. Can diagnostic delay be utilized as a key variable for monitoring the pool of infectious TB in a population? (submitted).

V. Storla, DG, Kristiansen I, Oftung F, Korsvold GE, Gaupset M, Gran G, Øverby AK, Dyrhol-Riise AM, & Bjune GA. Use of Interferon Gamma-Based Assay to Diagnose Tuberculosis Infection in Health Care Workers after Short Exposure (submitted).

SUMMARY

Since the 1980ies a worldwide resurge of TB has taken place. There are indications that the current TB-situation represent a new pandemic of emerging successful strains, and that it may take a different course from that observed previously, due to both host and parasite changes. This thesis aimed at contributing to the current knowledge that forms the fundament for local, national and international control of tuberculosis.

Fingerprinting (Paper I): A total of 111 *M. tuberculosis* isolates from new pulmonary tuberculosis (TB) patients, living in the rural Sunamganj district in northern Bangladesh were characterized with IS6110 restriction fragment length polymorphism analyses and spoligotyping at the National Reference Laboratory for Mycobacteria at the Norwegian Institute of Public Health (NIPH). Only 3 of the isolates belonged to the Beijing genotype of *M. tuberculosis*. A high degree of diversity indicated that the spread of *M. tuberculosis*, in this rural area, was not caused by closely related genotypes but rather represented a well established epidemic. The TB cases in the current study were less likely to represent recent transmission than what is commonly observed in the urban parts of south-east Asia. A majority of the strains belonged to the ancient East African-Indian (EAI) lineage, and the study was the first to describe the EAI6 BD1 sub-clade. More importantly, the different epidemic situation between urban and rural Bangladeshi areas became obvious. Previous studies had demonstrated the abundance of the Beijing lineage of *M. tuberculosis* in Dhaka [4], yet in the current study the EAI represented a well established epidemic. It was indicated that the TB cases of this isolated area in a high-incidence country, represented those of an established epidemic, not yet influenced by recently disseminated strains.

Susceptibility testing (Paper II): Spread of drug-resistant TB threatens TB-control programs, and all countries need to monitor the patterns and trends of anti-TB drug resistance. Such data assess the quality of control programs and help forecast future trends of drug resistance. It may also help establish guidelines for TB therapy in given settings. Among the current collection, 95 isolates of *M. tuberculosis* represented those from sputum-smear microscopy positive (ss+) patients. These isolates were

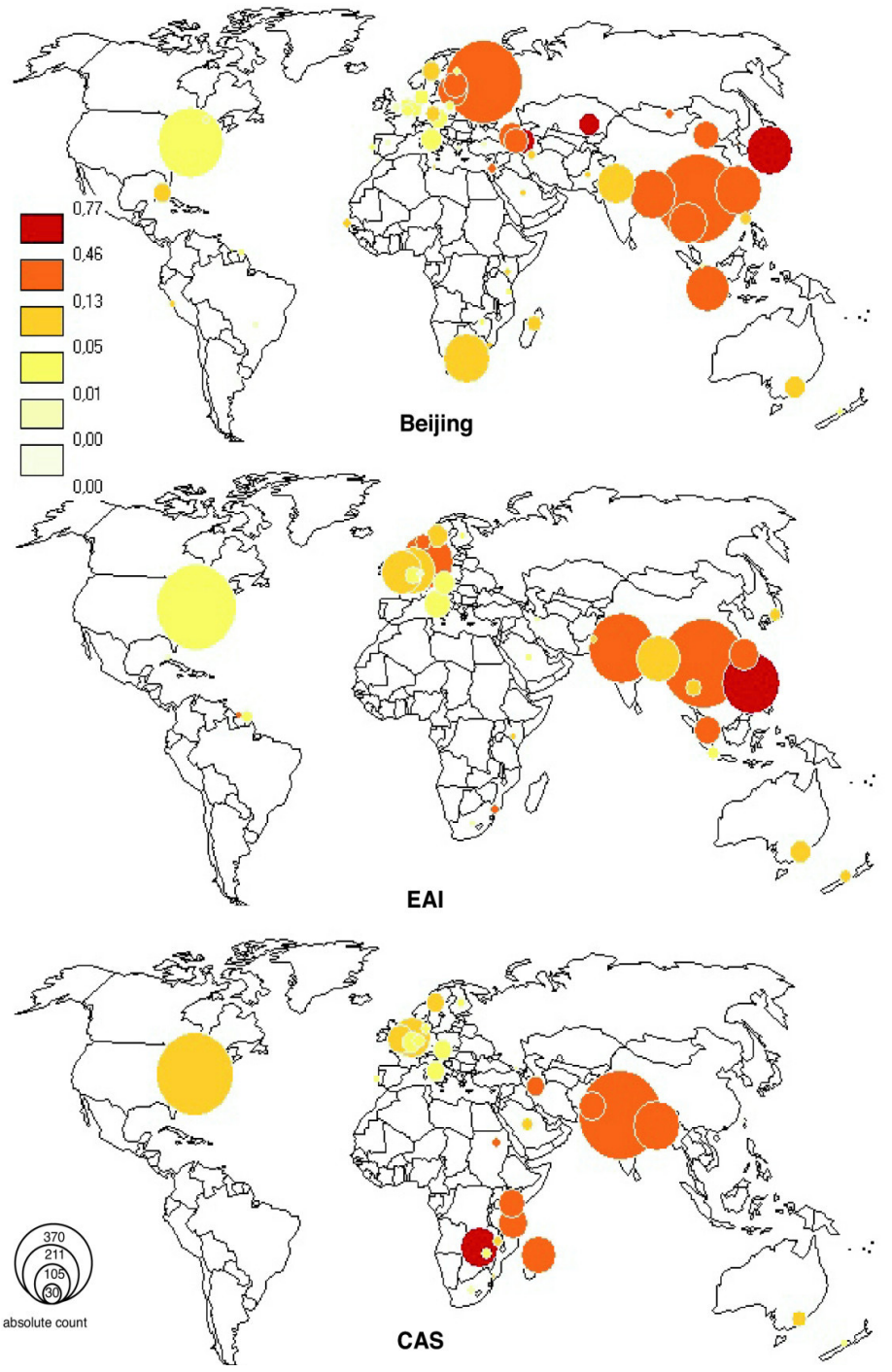
tested for susceptibility to streptomycin (SM), isoniazid (INH), rifampicin (RMP) and ethambutol (EMB) at the NIPH. The total resistance among new cases to any drug was 31%. For SM it was 18%, INH 23%, RMP 2%, EMB 10% and 2% were multidrug-resistant (MDR). The National Tuberculosis Program (NTP) in Sunamganj is still effective, although the high resistance to INH is alarming. An increased risk of treatment failure has been demonstrated in areas with high levels of INH resistance, and a high proportion of INH resistant cases may develop resistance to RMP during treatment.

Diagnostic Delay (Paper III): Early diagnosis and immediate initiation of treatment are essential for an effective TB control program. Delay in diagnosis is significant to both disease prognosis at the individual level and transmission within the community. Most transmissions occur between the onset of cough and initiation of treatment. A review of 58 studies addressing delay in diagnosis and treatment of TB was performed. We found different definitions of, for example, debut of symptoms, first appropriate health care provider, time to diagnosis, and start of treatment. Rather than excluding studies that failed to meet strict scientific criteria (like in a meta-analysis), we tried to extract the "solid findings" from all of them to arrive on a more global understanding of diagnostic delay in TB. The main factors associated with diagnostic delay included human immunodeficiency virus; coexistence of chronic cough and/or other lung diseases; negative sputum smear; extrapulmonary TB; rural residence; low access (geographical or sociopsychological barriers); initial visitation of a government low-level healthcare facility, private practitioner, or traditional healer; old age; poverty; female sex; alcoholism and substance abuse; history of immigration; low educational level; low awareness of TB; incomprehensive beliefs; self-treatment; and stigma. The core problem in delay of diagnosis and treatment seemed to be a vicious cycle of repeated visits at the same healthcare level, resulting in non-specific antibiotic treatment and failure to access specialized TB services. Once generation of a specific diagnosis was in reach, TB treatment was initiated within a reasonable period of time.

A systematic recording of diagnostic delay can be utilized as a tool to monitor the infectious pool of tuberculosis in a population (Paper IV): One out of two goals of TB control programs is to stop transmission of *M. tuberculosis*. However, this can not be rapidly accomplished, because in endemic areas most of the population is already infected, serving as a reservoir that continuously contributes to the pool of in-

fectious cases. Tuberculin surveys are the main tools used to monitor the infectious pool, but there are serious methodological constraints, and they require resources and expertise that are often unavailable. There is an urgent need for alternative means to monitor the epidemic at the local level. We investigated if a systematic registration of treatment delay in the tuberculosis program records of the Amhara Region of Ethiopia could be utilized to estimate the infectious pool of TB. By recording the treatment delay for new TB cases, retreatment cases and failures, and by estimating the number of undiagnosed cases, the total number of infectious days and hence an estimate of the infectious pool could be calculated. Of these categories, new sputum smear-positive TB cases contributed the greatest number of infectious days. A local TB program can use a systematic recording of treatment delay as a quantifiable variable to monitor the infectious pool, and can also serve as an indicator of program performance.

Interferon-gamma based Assay in the Diagnosis of tuberculosis infection after short-time Exposure of Health Care Workers (Paper V): Substantial resources are utilized to follow up personnel after unprotected exposure to patients with sputum-smear positive pulmonary tuberculosis in Norwegian health care institutions. Due to the low specificity of the Tuberculin Skin Test (TST), a large proportion of the group defined as super-infected after exposure are probably false positives, which may lead to incorrect treatment, waste of resources, and unnecessary anxiety. However, recently developed interferon-γ release assays based on the *M. tuberculosis* specific ESAT-6 and CFP10 antigens may improve specificity significantly. A total of 155 health care workers exposed to TB at three Norwegian hospitals as well as a non-exposed control group of 48 individuals were tested with both TST and the interferon-γ test T-SPOT.*TB*. Within the exposed group 42 individuals (27 %) were TST positive, while no more than 5 (12 %) of these had a positive T-SPOT.*TB* test. However, only 27 individuals were new TST positives after recent exposure, of whom 3 (11%) demonstrated a positive T-SPOT.*TB* test. All individuals in the control group were T-SPOT.*TB* negative, but three persons were found TST positive. Our data indicate that the frequency of latent TB in the total cohort of health care workers is low, as is the rate of transmission of TB to exposed individuals.



Synthesizing World Maps showing absolute (diameter) and percentage (colour) numbers of 3 genotype families within each country: Beijing; EAI (East-African Indian) CAS (Central Asia). These maps were built on an updated SpolDB4 on 2005 September 14th, on clusters of the 50 most frequent shared types for a total of n = 17212 isolates (Beijing n = 4042, EAI n = 1684, CAS n = 1022). Brudey *et al. BMC Microbiology* 2006

1. INTRODUCTION

1.2 THE GLOBAL EMERGENCY

Since the 1980ies a worldwide resurge of tuberculosis (TB) has taken place. There are indications that the current TB-situation represent a new pandemic and that it will take a different course from that observed previously, due to both host and parasite changes.

A family of *M. tuberculosis* that later received great attention was first described among isolates from the Beijing region of China. This particular genotype was demonstrated in >80% of the TB patients there in 1995 [7]. Soon reports of dominance of strains belonging to the Beijing Lineage (BL) appeared from various parts of the world (above map). For example in the Archangel oblast, Russia (1998-1999), Toungoussova et al. found that 44.5% of the strains belonged to the BL [8], and in the prisons 76% of the isolates were assigned to BL [9]. A total of 43.4% of the isolates of the BL were multidrug-resistant (MDR), compared to 10.6% MDR among the traditional strains. A total of 92.5% of the BL were part of a cluster, compared to 33.3% of the traditional strains. A higher rate of clustering and also a younger age among the BL strain patients indicated a higher degree of recent transmission. BL infections were also more commonly isolated from alcohol abusers and from patients with chronic obstructive lung disease. In 1990-93 strains of the “W-family” caused a large outbreak of drug resistant TB in New York [10]. It was later demonstrated that the “W-family” was identical to the BL. In this context it must be remembered that other *M. tuberculosis* lineages dominate in other parts of the world, and are related to clustering, drug resistance and other virulence related characteristics [11].

It has been emphasised that the BL is a heterogeneous group, and the clinical presentations vary between different geographical settings, featuring variable degree of virulence, drug resistance, host populations and clustering [12-16]. Currently, it is not clear whether the observed variability is a function of the BL population of particular geographical settings, or a function of the genetic composition of the human population, or a combination of these two variables. To explain the key role of the BL in the current global pandemic three main qualities of this lineage of strains have been outlined:

Firstly, there is increasing evidence that a majority of BL strains have increased virulence compared to other lineages [16]. Compared to other common *M. tuberculosis* families there are studies indicating that members of the BL has an increased expression of the phenolic glycolipid PGL-tb associated with a less efficient T helper cell 1 (Th1) response [17, 18]; a preference of inducing interleukin-4 (IL-4) and IL-13, which characterize Th2 polarized immunity (non-BL induce more IL-12 and INF-Y associated with phagocyte activation and Th1 protective immunity) [15, 19]; a decreased apoptosis of infected macrophages (Mφs) (associated with protection) and increased apoptosis of Th1 cells (associated with aggravation) [20]; an increased ppe44 expression associated with a higher virulence [21]; an increased expression of alpha-crystallin and decreased expression of Hsp65, PstS1, and the 47 kDa, all associated with increased virulence [22].

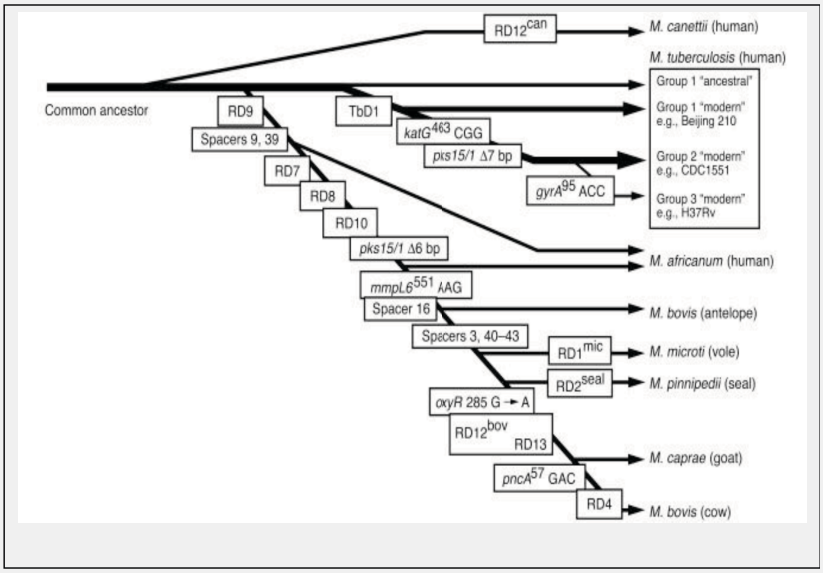
Secondly, as in Archangel and many (but not all) geographic areas where BL strains are frequent, they more frequently possess drug resistance than non-BL strains. A review of studies on the worldwide occurrence of BL found associations with drug resistance to be variable: only 4 of 12 studies reported statistically significant increases in the proportions of drug resistance among Beijing strains [16]. Among the Asian studies only one found a statistically significant increase in drug resistance in BL [16], and in Hong Kong BL were less likely than the others to be isoniazid resistant [23]. In New York, Cuba, and Estonia BL were strongly associated with drug resistance [24-26]. In given areas some members of the BL are strongly associated with resistance in not previously treated individuals, and cause clusters ten times as big as other strains [16, 27]. It is known that many bacteria develop drug resistance at the cost of fitness [28]. Drug resistant strains that experience competitive pressure from susceptible strains may loose their advantage if the selective antibiotic is no longer present in the environment. In the absence of the drug, the susceptible bacteria may have a survival advantage because they do not have to spend energy to circumvent the drug block in their metabolism. After a time, then, they may out-compete the resistant microbes. However, some RMP resistant strains of the BL exhibit similar growth rates as other sensitive BL [29]. Furthermore, fitness cost associated with the evolution of resistance to rifampin in *M. tuberculosis* may be different in clinical isolates compared to in vitro-generated mutants. An atypical Beijing strain demonstrated the ability to spread despite acquiring resistance to RPM. Transmission was linked to human immunodeficiency virus coinfection, raising concern for the spread of drug resistance in

vulnerable populations [30]. Another study found that BL strains have an increased acquisition of drug resistance and rapid adaptation to the environment as the consequence of increased single-nucleotide polymorphisms (SNPs) in the mismatch repair genes [31].

A well organised TB control program with a high level of treatment completion may control epidemics of *M. tuberculosis*, whether they belong to the BL or not [16]. Unfortunately, TB control programs in some high-incidence countries apparently do not receive necessary political commitment. Microbiological services are weak in many areas with high rates of TB. Primary drugs are often used in cases that are already resistant, because drug susceptibility tests are not available. Such practices accelerate the development of resistant *M. tuberculosis* populations and may eventually lead to epidemiological situations of catastrophic dimensions.

Third, recent studies from Vietnam indicate that *Mycobacterium bovis* BCG does not induce protective immunity towards the BL [32, 33]. Experimental animal studies from Mexico and USA indicate the same for given isolates of the BL [34-36]. *M. bovis* BCG still represent the only available vaccine against TB, if escape variants of *M. tuberculosis* exist the urgency of the global TB situation is clearly underscored.

Scheme of the evolutionary pathway of the tubercle bacilli illustrating successive loss of DNA in certain lineages



Brosch et al. (2002). With kind permission from Proc. Natl. Acad. Sci.

1.3 THE EVOLUTION OF *M. TUBERCULOSIS*

1.3.1 TB evolution and aetiology

Humans have been challenged by mycobacterial infections for thousands of years. It is believed that the genus *Mycobacterium* originated more than 150 million years ago [37]. Communicable diseases require host populations that comply with their required critical community size (CCS). The CCS is the number of potential hosts that need to live in a community if an infectious disease is to exist within it. Although not yet epidemic, an early progenitor of *M. tuberculosis* was probably infecting hominids in East Africa three million years ago [6]. It has been calculated that the currently known members of the *M. tuberculosis* complex (MTC) originated from a hypothetical common progenitor about 15,000 - 35,000 years ago [6]. It is commonly believed that this bacterial evolution coincided with the establishment of agricultural communities and that communicable *M. tuberculosis* found its CCS in the early human societies.

Tuberculous skeletal lesions have also been identified by archaeologists at various sites all over the world [38].

The genus *Mycobacterium* is divided into two main groups. The MTC and non-tuberculous mycobacteria (NTM), often called environmental mycobacteria [39]. The NTM group is genetically heterogeneous, and include the *M. avium* complex as well as a wide range of other species. They can be isolated from environmental sources like soil and water, are usually non-pathogenic for immuno-competent humans, and are often the source of false positive tuberculin skin tests (TST), which can be defined as e.g. a TST > 10 mm not caused by members of the MTC.

The species of the MTC include *M. tuberculosis*, *M. bovis* (including the attenuated BCG strains), *M. microti*, *M. canettii* and *M. africanum* [40]. It was long believed that *M. tuberculosis* developed as a result of *M. bovis* crossing the species barrier from cattle to humans, after humans domesticated wild animals. Recent phylogenetic studies however, show that 20 known regions of difference (RD) did not occur as results of spontaneous site mutations, but rather represent ancient, irreversible genetic events that apparently have taken place in common progenitor strains [40]. The structure of the phylogenetic tree is mainly based on deletions rather than mutations and gene acquisitions. Based on the presence or absence of a *M. tuberculosis* specific deletion (TbD1), the strains are divided into “ancestral” or ‘‘modern’’. Because the original habitat of *Mycobacteria* apparently was soil or water a number of genes needed for these environments became “surplus” as the main hosts ended up to be humans and other mammals. The evolution can bee seen as a comprehensive process to delete these no longer useful genes [6]. Wirth et al. obtained new insights into the origin of MTC and its dynamic association with the human host. By using mycobacterial tandem repeat sequences as genetic markers, they showed that MTC consists of two independent clades; *M. tuberculosis* lineages from humans, and *M. tuberculosis* lineages from both animals and humans. The latter was also likely derived form a human pathogenic lineage, supporting the hypothesis of an original human host. They provided important genetic evince indicating that the most common ancestor of the bacterial complex emerged some 40,000 years ago from its progenitor in East Africa, the region from where modern human populations disseminated around the same period [41].

Based on analysis of partial gene sequences of *katG* and *gyrA,* Sreevatsan et al. defined three subgroups among the “modern” strains of *M. tuberculosis.* Group

1 include the BL and have the *katG*^{463} CTG (Leu) and *gyrA*^{95} ACC (Thr) mutations, Group 2 that include the Haarlem lineage and Africa clusters carry a *katG*^{463} CGG (Arg) mutation, in addition to the *gyrA*^{95} ACC (Thr). Group 3 that include the H37Rv strain also carry a *katG*^{463} CGG (Arg) mutation but also exhibit a *gyrA*^{95} AGC (Ser) mutation [42]. In "ancestral" strains, where the TbD1 region is not deleted, Leucine (CTG) is found at *katG*^{463}. It is possible that during the evolution of *M. tuberculosis*, the *katG* mutation at codon 463 (CTG (Leu) → CGG (Arg)) occurred in a progenitor strain that had already deleted the TbD1 [40, 43].

The evolution of *M. africanum*, *M. microti*, and *M. bovis* have been found to be the result of the loss of DNA in the Region of Difference 9 (RD9) that diverged from the hypothetical progenitor strain prior to the deletion of the TbD1. It is therefore unlikely that *M. tuberculosis* evolved from *M. bovis*. Since *M. canettii* and ancestral *M. tuberculosis* strains harbour both TbD1 and RD9 it is reasonable to believe that they represent descendants of tubercle bacilli that existed prior to the divergence of the *M. africanum/M. bovis* lineage. Principally, the only thing we know about "ancestral" strains is that they no longer exist, but we can anticipate that the common ancestor of the tubercle bacilli resembled *M. tuberculosis* or *M. canettii* and was possibly already a human pathogen [40].

1.3.2 Molecular biology and epidemiology

According to 16S rRNA studies the genus *Mycobacterium* belongs to the actinomycete branch of Gram-positive bacteria. Typically, mycobacteriae unlike other members of the group lack a spore stage in the life cycle. The majority of the actinomycetes are soil-dwelling and non-pathogenic organisms. *M. tuberculosis* also probably lived in the soil before it was specialized to its human host [38, 44].

In 1998 the complete genome sequence of *M. tuberculosis* H37Rv was published [45], and later the genomes of several other strains have been sequenced. Utilizing microarrays, Behr et al. identified 14 regions (RD1-14) that were present in H37Rv but absent in *M. bovis* BCG Pasteur, and two regions (RD15 and 16) that were found in only some BCG strains. The deleted regions included many genes of unknown functions but also genes that coded for several known proteins such as the ESAT-6 secreted antigen and CFP-10 phospholipase C [46]. *M. tuberculosis* was found to have 4,411,529 bp on the circular genome that contains 3,924 open reading frames. Based on similarity to known genes, 40% had confidently assigned functions, 44% a probable function and 16% were left as orphan genes with no known function in 1998 [46].

M. tuberculosis has more than 250 genes devoted to lipid and polyketide metabolism, suggesting that it has a lipolytic lifestyle in vivo. Before entering into the dormant state, *M. tuberculosis* accumulates lipids originating from the host cell membrane. When reactivated, these lipids are hydrolysed and they start to multiply. In active disease, degradation of both extra and intracellular lipids is a key issue, which was ignored for a long period due to difficulties in obtaining high expression levels of lipolytic enzymes. The completion of the *M. tuberculosis* genome offered a new opportunity to these kind of studies [47].

Surprisingly, the genome also contains several genes encoding for enzymes needed for anaerobic metabolism like nitrate- and fumarate reductase, which is unexpected considering the aerobic nature of *M. tuberculosis*. The explanation is that when oxygen is slowly depleted from growing cultures of *M. tuberculosis*, they enter a dormant state seen with latent TB. Sohaskey et al. found that in this hypoxic state, nitrate reductase activity is strongly induced [48].

About 10% of the genome codes for 99 members of the Proline-Glutamic acid motif (PE) protein family and 68 members of the Proline-Proline-Glutamic acid motif (PPE) family. The genes coding for these families contain polymorphic repetitive se-

quences, PGRSs (PE) and polymorphic tandem repeats, PMTRs (PPE). Their function is mostly unknown, but it is believed that they are involved in generating antigenic variation and disease pathogenesis. There is evidence that the expansion of the PE and PPE gene families is linked to the duplications of the ESAT-6 gene clusters. Members situated in and associated with ESAT-6 represent the most ancestral copies of the PE and PPE gene families. Their emergence is a recent evolutionary event [49], occurring at defined branching points in the evolution of the *Mycobacterium* genus, and are only present in multiple copies in the members of the MTC and close relatives [49].

Several proteins have been identified that may act as potential virulence factors including phospholipases C [50], lipases and esterases [51]. Proteins involved in metabolism and storage of oxygen, carbon and iron have also been identified, and seem to be associated with pathogenetic processes that facilitate the entry, replication, and persistence of *M. tuberculosis* in its host [52].

There are four copies of the mycobacterium cell entry (*mce*) operon in the genome. The *mce* gene was originally characterized as a Mφ-colonizing factor in *M. tuberculosis.* Six mammalian cell entry proteins (*Mce3*A-F) encoded by the *mce3* operon are expressed during in vitro growth of *M. tuberculosis* [53].

1.3.3 Molecular fingerprinting methodologies utilized for M. tuberculosis

As long as TB is not under control, it becomes increasingly urgent to monitor the epidemiology both at regional, national and local levels. Knowledge on the epidemiology of *M. tuberculosis* is crucial in order to control the disease. Analysis of TB transmission patterns and other aspects in TB epidemiology improved tremendously after the introduction of DNA fingerprinting of *M. tuberculosis*. Until the 1990s, antibiotic susceptibility patterns and phage typing were used for *M. tuberculosis* strain identification, but these methodologies have significant limitations. The discovery that the patterns of certain transposable elements in the *M. tuberculosis* genome can be utilized for strain identification has proven to be superior to the more traditional techniques, particularly in outbreak investigations [9, 54]. Repetitive DNA elements in *M. tuberculosis* were recognized independently by Eisenach [55] and Zainuddin and Dale [56].

The most widespread and robust genotyping tool, used to discriminate or subspeciate clinical isolates of *M. tuberculosis* is the insertion sequence (IS) *6110* restriction fragment length polymorphism (RFLP) [57]. By use of IS*6110* RFLP one can type the *M. tuberculosis* isolates on the basis of the number and position of IS*6110* [57]. In our Sunamganj study IS*6110* RFLP was utilized as the main fingerprinting tool.

Spoligotyping is based on the in vitro amplification of the DNA of the highly polymorphic Direct Repeat (DR) genomic locus present in the *M. tuberculosis* chromosome. In our study spoligotyping was used to differentiate isolates that carried less than 5 copies of IS*6110* and to identify members of the various *M. tuberculosis* lineages [58].

The main description of these two methodologies is found in section 2.2.6.

OTHER FREQUENTLY USED FINGERPRINTING METHODS

MIXED-LINKER PCR

DNA is digested with the *Hha*I restriction enzyme, and a double-stranded oligonucleotide linker is ligated to the ends of the restriction fragments. The name “mixed linker” comes from the use of two oligonucleotides where uracil is substituted for thymidine. This step is followed by PCR with an IS*6110*-specific oligonucleotide primer and a linker primer. The sequence of the linker primer is identical to that of the thymidine-containing strand of the linker except that it lacks the two nucleotides complementary to the 3' overhang created by restriction with *Hha*I. The main advantage of mixed-linker PCR fingerprinting over the traditional RFLP method is its independence from mycobacterial growth, where RFLP requires 2-3 weeks of cultivation. A fingerprint could be obtained directly from a single colony of the primary isolate without further culture. In an outbreak situation, a specific RFLP type should correlate well with the drug resistance pattern of the isolates, and rapid typing of the isolate might be used to design the treatment regimen [59].

A further development of this method, called fast ligation-mediated PCR (FLiP) allows analysis of strains within one working day, and starts from less than 1 ng of mycobacterial DNA or a crude cell lysate. Blinded analysis showed the ability to differentiate 81 types among 90 *MTC* isolates with 84 different IS*6110* RFLP patterns. FLiP is suggested to serve as a method to rapidly detect chains of transmission prior to starting time-demanding standard IS*6110* RFLP [60].

MIRU-VNTR

Based on the above mentioned polymorphic tandem repeats of the PPE region the variable-number tandem repeats fingerprinting methodology (MIRU-VNTR) has been developed. Twelve mycobacterial interspersed repetitive unit (MIRU) loci were initially amplified and the amplicons were analyzed by agarose gel electrophoresis to determine the copy number at each MIRU locus. In some studies MIRU-VNTR has produced more distinct patterns than IS*6110* RFLP or spoligotyping. [61].

AFLP and FAFLP

Amplified fragment-length polymorphism (AFLP), or its fluorescent version FAFLP, is a PCR-based fingerprinting technology. The first step of AFLP/FAFLP is to cut the whole *M. tuberculosis* genome into fragments utilizing restriction enzymes. The next step is ligation of adaptors complimentary to the restriction sites and selective PCR amplification of a subset of the adapted restriction fragments. These fragments are finally visualized on denaturing polyacrylamide gels either through autoradiographic or fluorescence methodologies (FAFLP). The availability of many different restriction enzymes and corresponding primer combinations provides flexibility, enabling AFLP/FAFLP to be utilized for a multitude of tasks like polymorphism screening, quantitative trait locus (QTL) analysis and genetic mapping. Although conflicting experiences exist (Ulf R. Dahle, personal communication), compared to other fingerprinting methods some authors report that AFLP/FAFLP provides equal or enhanced performance in terms of reproducibility and resolution [62, 63].

Reproducibility and number of types obtained by using various DNA typing methods for differentiation of mycobacterial strains

DNA target	Method used [a]	Reference	Reproducibility (%) [b]	No. of types obtained
IS*6110*	RFLP (*Pvu*II)	(van Soolingen 1994)	100	84
IS*6110*	Mixed-Linker PCR	(Haas 1993)	100	81
IS*6110*	FLiP	(Reisig 2005)	97	81
IS*6110*	IS*6110* inverse PCR	(Otal 1997)	6	nd [c]
IS*6110*	LM-PCR	(Prod'hom 1997)	81	73
IS*6110*/MPTR	IS*6110* ampliprinting	(Plikaytis 1993)	39	nd
IS*6110*/PGRS	DRE-PCR	(Friedman 1995)	58	63
15 loci	VNTR typing	(Supply 2006)	nd	89
12 MIRUs	VNTR typing	(Supply 2001)	100	78
ETRs A-E	VNTR typing	(Frothingham 1998)	97	56
5 QUBs [d]	VNTR typing	(Roring 2004)	87	82
DR locus	Spoligotyping	(Kamerbeek 1997)	94	61
DR locus	2nd gen. spoligotyping	(van der Zanden 2002)	90	61
DR locus	RFLP (*Alu*I)	(van Soolingen 1993)	100	48
PGRS	RFLP (*Alu*I)	(van Soolingen 1993)	100	70
(GTG)5	RFLP (*Hin*fI)	(Wiid 1994)	94	30
Total genome	APPCR	(Palittapongarnpim 1993)	71	71
4 conserved loci	Amadio PCR	(Amadio 2005)	74	13
*EcoR*I/*Mse*I sites	FAFLP typing	(Ahmed 2003)	7	nd
*EcoR*I/*Mse*I sites	FAFLP typing	(Sims 2002)	0	nd
*BamH*I/*Pst*I sites	FAFLP typing	(Kremer 2005a)	0	nd

[a] RFLP; Restriction Fragment Length Polymorphism, FLiP; Fast Ligation Mediated PCR, LM-PCR; Ligation-Mediated PCR, DRE-PCR; Double Repetitive Element PCR, VNTR; Variable Numbers of Tandem Repeats, APPCR; Arbitrarily Primed PCR, FAFLP; Fluorescent Amplified Fragment Length Polymorphism.

[b] Fraction of duplicates showing identical types (31)

[c] nd, not done

[d] Results indicated exclude QUB locus 3232

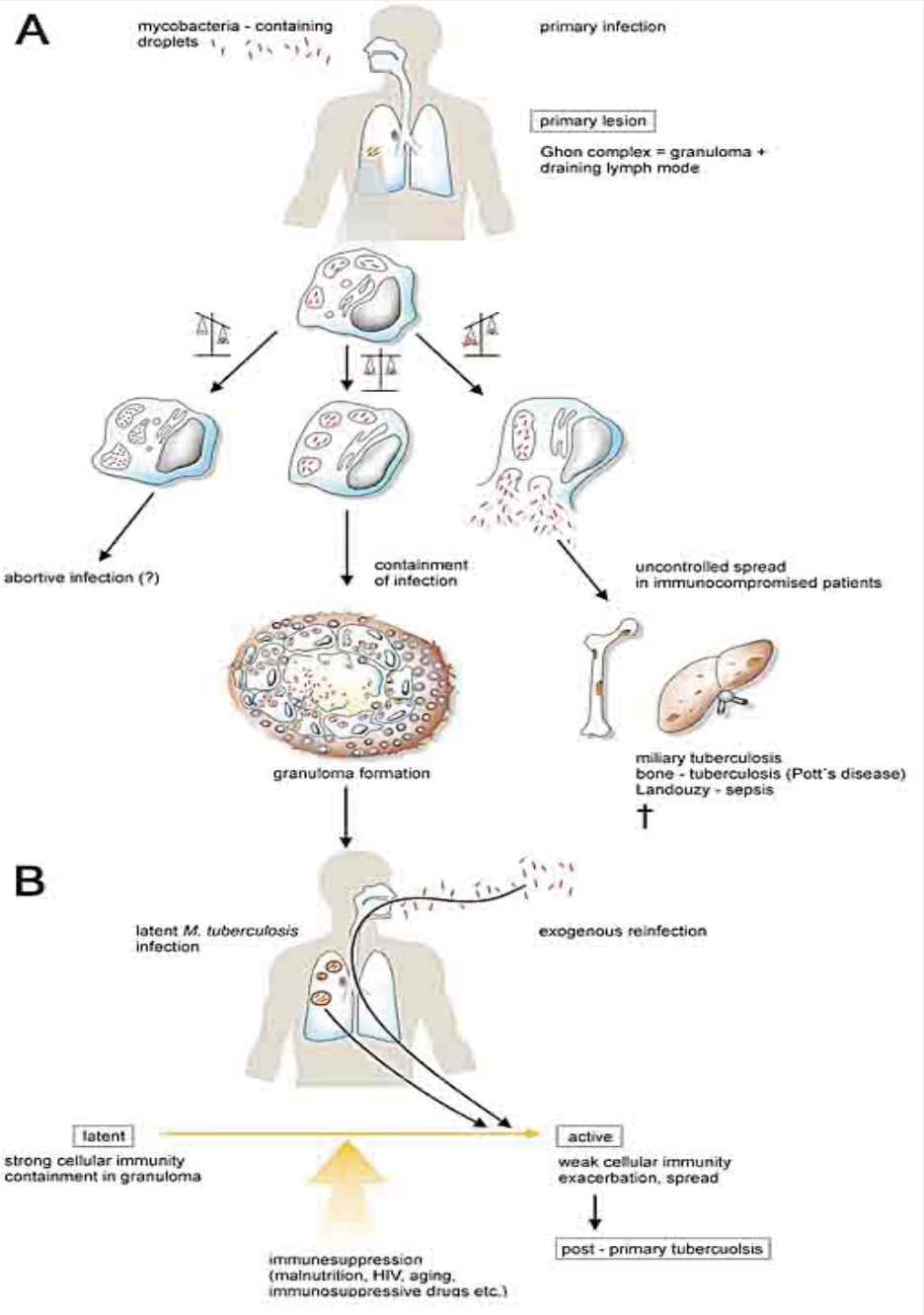
Kremer 1999 [5], Kremer 2005a [6]

As molecular fingerprinting was introduced, basic assumptions had to be changed. By identifying identical fingerprints, transmission could be traced from case to case and outbreaks with clusters of identical fingerprints could be discovered. It was for example previously thought that a majority of TB cases in Western Europe come from reactivation of remote infection [64]. Using fingerprinting, de Viedma et al. found that 33% of patients with reoccurring TB in Spain was due to reinfection with a new strain rather than reactivation [65]. Another example is the intensified effort to control the rapid spread of MDR-TB. To identify the strain patterns and thus be able to analyze the patterns of transmission is a basic need of a good TB control program. Generally, DNA-fingerprinting is useful to

1. identify laboratory contamination
2. Identify and decipher outbreaks
    - differentiate between reactivation and reinfection
    - determine risk factors for transmission
    - evaluate preventive measures
3. perform surveillance of transmission, both nationally and internationally

These three usages of DNA-fingerprinting are not further described in the text, as this would demand voluminous space and is beyond the scope of this thesis.

1.4 THE HUMAN HOST



The human host's response to infection with *M. tuberculosis*

From Kaufmann and Hahn: Mycobacteria and TB. Karger 2003 (with permission)

1.4.1 Pathology and Natural history of infection

Fortunately, the human immune system generally controls efficiently the vast majority of infections. As illustrated above, in the case of TB there are three possible outcomes of the primary, early infection [66]:

1. *M. tuberculosis* is eradicated; abortive infection. This is a mechanism that has yet not been proven, but probably accounts for a proportion of infected people.
2. Containment of infection, or the establishment of latent infection; accounts for >90% of the cases.
3. Primary TB.

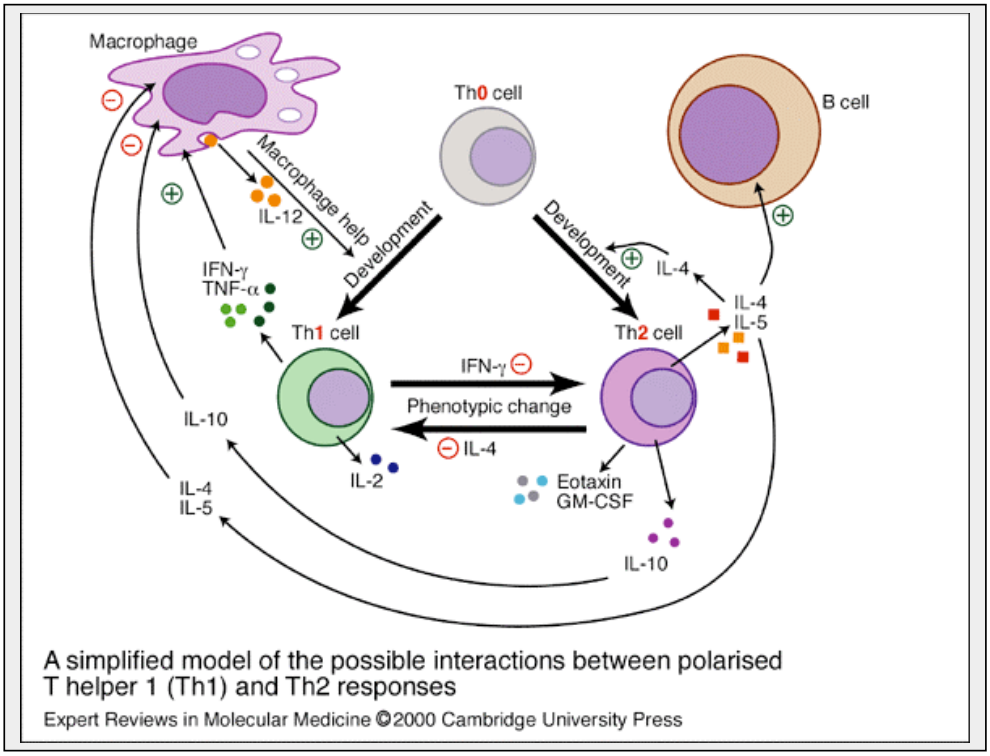
Infection with *M. tuberculosis* is the result of inhalation of airborne droplet nuclei that are generated when individuals with active pulmonary TB cough [67]. On their way through the bronchial tree, the droplets have to pass several kinds of physical barriers like the nasal conches and the ciliated and delicately distributed bronchial tree before a few bacilli finally may reach the alveoli and are engulfed by the alveolar Mφs, and subsequently as they spread through the lymphatic system, the bacilli are also engulfed by dendritic cells in the regional (mediastinal and hilar) lymph nodes, where the major part of antigen presentation takes place [68].

The immune response seems to be depending on the type of receptor *M. tuberculosis* utilizes to enter the host cell. Entry of IgG-opsonized mycobacteria via FcR receptors results in activation [69], while entry via complement receptor 3 (CR3) does not activate the cell properly [70]. Other receptors that take part in the engulfment is the Mφ mannose receptor (MMR), surfactant protein receptors, cluster of differentiation 14 (CD14) pattern recognition receptor, and toll-like receptors 2 and 4 (TLR2 and TLR4) [71].

However, also neutrophils play an active role in the first innate immune response. The idea that neutrophils are able to kill mycobacteria is supported by some studies [72], but not by others [73]. But the function of neutrophils goes beyond their microbicidal ability. It is believed that they contribute to the control of infection through the production of chemokines [74], take part in the induction of granuloma formation [75] and transference of their own microbicidal agents such as human neutrophil α-defensins and cathelicidins to infected Mφs [76].

As fragments of *M. tuberculosis* peptides are presented to T helper cells through the major histocompatibility complex II (MCH II) on the surface of the Mφs or dendritic cells, a strong, specific Th1 response of cell-mediated immunity is triggered.

The indifferent T helper cells (Th0) are stimulated to become Th1 cells under the influence of signal substances like interleukin 12 (IL12) that are secreted by activated Mφs. On stimulation, Th1 cells excrete interferon-γ (INF- γ), interleukin 2 (IL2) and tumour necrosis factor-α (TNF-α) that further reinforce a strong, cell-mediated Th1 immune response [77]. Th1 type responses are essential to protection against intracellular parasites. In contrast, Th2 responses, which are characteristic of allergic disorders and helminth infections, are rather believed to be responsible for immunopathology, and thus active disease for infections with intracellular pathogens [78]. The current view is that TNF-α/IFN-γ activated Mφs are able to effectively eliminate intracellular pathogens, and that IL-4 and IL-10 suppress Mφ activation and thus parasite clearance. The different types of responses also negatively influence each other with IL-4 favoring the development of Th2 and IL-12 the development of Th1 type cells. The INF-γ produced by Th1 cells directly inhibits the development of Th2 type cells [79].



A simplified model of the possible interactions between polarised T helper 1 (Th1) and Th2 responses

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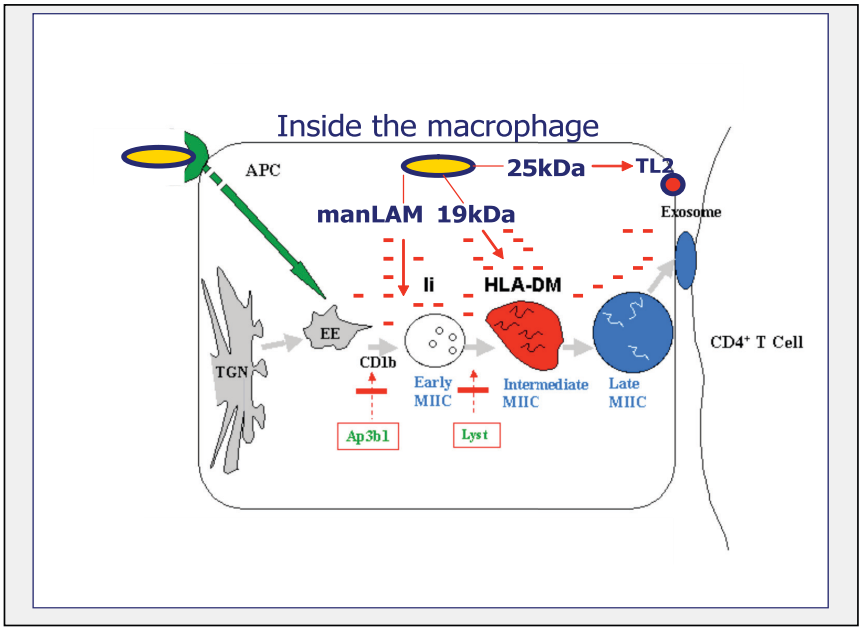
Ultimately, most *M. tuberculosis* bacilli are killed within the Mφs. But *M. tuberculosis* has developed a range of mechanisms to evade the immune response, and in some cases the Mφs fail to kill the bacilli. On the contrary, the Mφs become sanctuaries where *M. tuberculosis* survive and even multiply [80]. In the murine model of Mustafa et al. mycobacterial antigens were concentrated to 3-6 % of the Mφs in lung granulomas [81].

A crucial effector pathway of protective immunity is nitric oxide (NO) and its intermediates. It is both the major bactericidal substance of the lysozyme and has an important signalling and second messenger function. NO-knock-out mice that are infected with *M. tuberculosis* develop an aggravated disease. NO is transformed into highly cytotoxic reactive nitrogen intermediates (RNI) by the enzyme NOS2. *M. tuberculosis* actively blocks the action of NOS2, thus inhibiting the production of RNI [82].

The role played by the reactive oxygen intermediates (ROM) during infection has not been explained completely, though it is known that hydrogen peroxide produced by Mφs activated by cytokines has a mycobactericidal activity. Also, it has been found that the tubercle bacillus presents molecules such as lipoarabinomannam (LAM) and phenolic glycolipid I, which work as oxygen radical scavenger molecules [83].

Not only is the NO pathway inhibited. The term “fortress *M. tuberculosis*” has been used to describe the antioxidant complex of *M. tuberculosis*. The complex consists of substances that are actively secreted by *M. tuberculosis* to protect it self from being phagocytized, like dihydrolipoamide dehydrogenase, thioreductin-like AphD, NAD dependent peroxidase, dihydrolipoamid succinyltransferase, peroxinitrite reductase, dihydrolipoamid succinyltransferase, truncated haemoglobin and AhpC peroxiredoxin [84].

M. tuberculosis has also developed mechanisms to survive within the phagosome. Among the strategies is to inhibit the H^+-ATPase that pumps H^+ into the phagosome, thus making it less acidic and hostile [85, 86]. Another strategy is to inhibit the maturation of the phagosome by utilizing the surface polysaccharide mannose capped mannosylated lipoarabinomannam (ManLAM) to block the early-endosomal autoantigen 1 (EEA1), which binds phosphatidylinositol-3-phosphate, a Rabankyrin 5 (Rab5) effector that is required for endosome fusion [87]. The fusion of *M. tuberculosis* with the lysosome is also inhibited by modulating soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) proteins, thus inducing accumulation of tryptophan-aspartate containing coat protein (TACO) [85, 88]. When Mφs were made

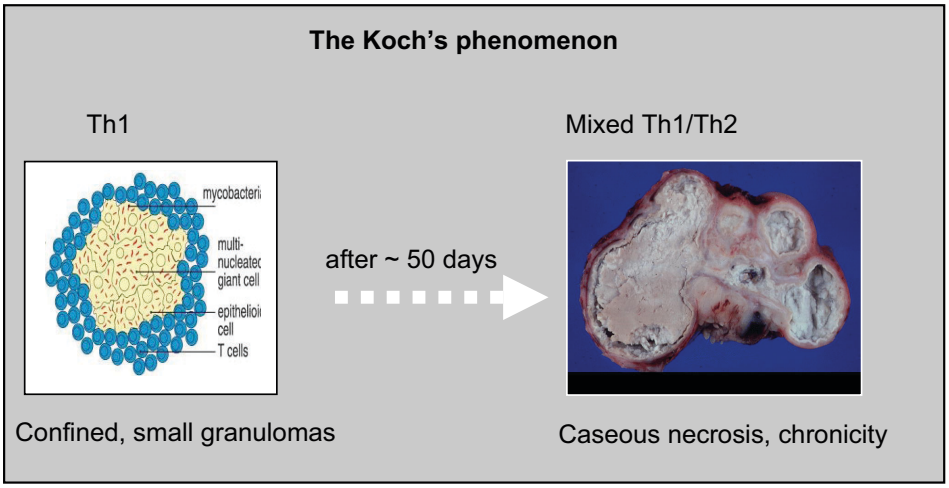


TACO-deficient experimentally, the maturation of mycobacterial phagosomes was not arrested and therefore these cells were able to eliminate bacilli by fusion of phagosomes with lysosomes [89]. TACO binds itself to the plasmatic membrane of Mφs through cholesterol, which also plays an important role in mycobacterial uptake by Mφs [90].

Another important defence mechanism is to avoid a specific immune response by inhibiting the expression of *M. tuberculosis* antigens through the MHC II complex on the surface of the Mφs. Two secreted peptides, one of 19 KDa and one of 25KDa, execute a direct inhibition of the Toll-like 2 (TL2) surface receptor, that is crucial to the development and excretion of the MHC II complex (named *exosome* in the above figure) [91].

There is also evidence that *M. tuberculosis* evades the immune system by choosing non-professional phagocytes as host cells. In a study of necropsy specimens from people who died from other reasons than TB, mycobacterial DNA was detected in Mφs, type II pneumocytes, fibroblasts, and endothelial cells [92]. Other studies have confirmed that endothelial cells [93] and fat cells [94] can host *M. tuberculosis* bacilli and allow them to replicate. On the other hand, epithelial cells also seem to be able to initiate a pro-inflammatory immune response, e.g. by secreting IL-8 [95] or inducing the production of NO [96].

As the TB granulomas matured in the murine model of Mustafa et al., they acquired a distinct morphology. However, there was a striking lack of lymphocytes surrounding the infected Mφs, and the frequency of apoptotic cells was high [80]. It is demonstrated that *M. tuberculosis* has evolved several evasion mechanisms to withstand the hostile environment of Mφs, but how does the infected Mφs escape from the cytotoxic T-cells? Mustafa et al. found that *M. tuberculosis* infected Mφs strongly expressed Fas ligand (FasL), indicating that a mycobacterial infection can induce an increased expression of FasL in the infected Mφs. Mφs, like almost all cells express Fas, and even if they fail to kill engulfed bacteria, the Mφs will express bacterial antigens, and thus become susceptible to cytotoxic lymphocytes. As *M. tuberculosis* induces the expression of FasL in the Mφs, apoptosis will be induced in the Fas-expressing sensitized lymphocytes. Thus, the epithelioid cells and the multinucleated giant cells (which are metamorphotic Mφs) by virtue of the increased expression of FasL may make the granulomas an immune privileged site for mycobacteria [97] .



1.4.2 The Koch's phenomenon

Among the first to study the pathogenesis of TB was Robert Koch, who inoculated guinea pigs with TB to establish infection. After 4-6 weeks he once again injected them intradermally with TB culture filtrate. He then observed what has later been named the Koch's phenomenon (KP); a necrotizing, inappropriate response pattern

[98], which is also the typical immunopathological response pattern in humans [99]. Studies of the human immune response to *M. tuberculosis* infection have demonstrated that almost all immunocompetent individuals start out with a strong, completely Th1-dominated cellular response that is comprehensive, and leads to the containment of the *M. tuberculosis* bacilli in confined granulomas, which is typical for latent infection. However, if the infection progresses to primary active disease, after approximately fifty days there is a marked shift towards a mixed Th1/Th2 response that results in a necrotizing lung tissue damage dominated by caseous necrosis, caverns and chronicity. The same pattern is seen in the case of reactivation of latent disease. It is not *M. tuberculosis* itself that causes the severe damage, but the human host's inappropriate immunological response to it [99]. There is evidence that pro-Th1 cytokines like IL-12, TNF-α and INF-γ may not only facilitate granuloma formation and bacillary elimination, but may also cause the local tissue necrosis typical of the KP, and systemic effects such as fever and wasting, due to the release of TNF-α into the circulation [100]. At the same time anti-inflammatory cytokines such as IL-10, tumor growth factor beta (TGF-β) and IL-4 down regulate the immune response and limit tissue injury by inhibiting an incomprehensive inflammatory response. These cytokines, if produced in excess, may result in failure to control infection resulting in widely disseminated TB. It is the balance between the pro-inflammatory and anti-inflammatory immune responses that determines the outcome of the TB infection. In the phase of the mixed Th1/Th2 an increased INF-γ as against reduced TNF-α probably suggests a better outcome [100]. In the murine model developed by Mustafa et al. a similar pattern of an initial asymptomatic phase with a pure Th1 pattern and stable bacillary growth followed by a mixed Th1/Th2 phase of severe necrotizing lung pathology was seen [77].

In the human host, once an inappropriate balance with local tissue necrosis/KP is established, the imbalance does not correct itself during the natural course of the disease. Without appropriate treatment half of those with active pulmonary TB die, and half of them survive with severe lung tissue damage [101]. The inappropriate mixed Th1/Th2 response also does not change back to the first comprehensive pure Th1 response during treatment. This is one of the reasons why the standard treatment has to go on for at least six months. If treatment is discontinued at 3 months, relapse rates of up to 20% can be expected, even if sputum smears have become negative well in advance, and there are few surviving bacilli [99]. Graham Rook has postulated that if we could learn how to pace the immune response back to the first,

pure Th1 pattern, ultra-shortcourse chemotherapy regimens would be possible. Such an immunotherapy supported shortcourse could drastically reduce the efforts needed to treat each case of TB, and for the first time in history give perspectives of controlling or even eradicating the disease [99].

1.5 TREATMENT AND DRUG RESISTANCE

1.5.1 Treatment

Chemotherapy for TB became available in the 1940s with the introduction of streptomycin. Gradually the duration of therapy decreased from 18 months to currently 6 months [102]. This happened in several steps: First the introduction of "triple therapy" (Streptomycin (SM or S), Para-aminosalicylic acid (PAS) and Isoniazid (INH or H)) in 1952, which assured cure. The next step forward took place in the 1970s when it was discovered that INH in combination with Rifampin (RMP or R) could reduce the duration of treatment from 18 to 9 months. The final step towards only 6 months treatment was introduced in the 1980s by adding Pyrazinamide (PZA or Z) and Ethambutol (EMB or E) during the first two months [103]. A four drug regimen of INH, RMP, PZA and EMB for two months followed by INH and RMP for four months (2HRZE/4HR) is currently the standard primary treatment drug regimen recommended by WHO [104].

The included anti-TB drugs have different abilities and capacities to kill bacte-

Anti-TB drugs and drug resistance mechanisms

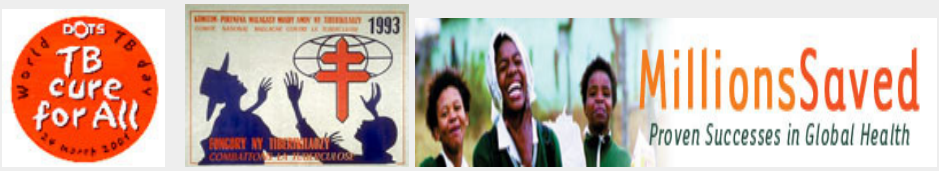
Site of Action	Antituberculous drug	Genes associated	Mutation frequency %
Inhibition of nucleic acid synthesis	Rifampicin	*rpo*B	>95
Inhibition of protein synthesis	Streptomycin	*rps*L, *rrs* (16S RNA)	52-59
	Amikacin/kanamycin	*rrs* (16S RNA)	80
Inhibition of cell wall synthesis	Isoniazid	*kat*G, *inh*A, *aph*C, *kas*A, *ndh*	33-37
	Ethionamide	*inh*A	34
	Ethambutol	*emb*CAB	39
	Cycloserine	*alr*A	46
Unknown	Pyrazinamide	*pnc*A	58

Adapted from Hatfull and Jacobs [1]

ria, to sterilize different kinds of human tissues and to prevent the emergence of drug resistance. INH acts on metabolically active bacteria, and is the most potent drug responsible for the major early bactericidal activities of the 2HRZE/4HR regime [105, 106].

RMP has a potent sterilizing activity, and is the backbone of the regime. It not only acts on rapidly dividing bacteria as INH, but it also kills “persisters” in intermittent periods of metabolism. It can be said that the other drugs are RMP’s “bodyguards”; their major task is to prevent the emergence of RMP resistance, and especially the combination of INH and RMP is effective [107].

PZA is also bactericidal, and has been added because it has a good sterilizing capacity by killing intracellular bacilli inside the Mφs in an acid environment. EMB and SM are less potent drugs, EMB is probably bactericidal in high doses [108].



The DOTS strategy comprises five components [2] :

(1) Political commitment for support to a strong national program.

(2) Case-detection through sputum smear microscopy of all suspects coming to the general health services.

(3) Directly observed treatment, with short-course therapy of all sputum smear-positive patients

(4) Regular and uninterrupted supply of anti-TB drugs.

(5) A monitoring system for evaluation of treatment outcome.

DOTS (Directly Observed Treatment, Short-course) has been presented as one of the most cost-effective health strategies available, and is both heavily supported by the World Health Organisation (WHO) and International Union Against Tuberculosis and Lung Disease (IUATLD) and utilized as the standard approach in most TB programs all over the world. DOTS implies that the drugs are taken by direct observation by a person that has been authorized by the NTP, usually a professional health care

worker. The DOTS strategy has three major goals: to treat patients already infected with TB, to prevent new infections and to avoid the development of drug resistance [109]. From 1995-2003 17 million patients were treated under the DOTS strategy, implemented by 182 countries. An estimated 77% of the world's population lived in regions utilizing DOTS with an average success rate (treatment completion) of 82% [110]. Globally, the rate of case detection, defined as the proportion of estimated new smear-positive cases notified by the NTP, reached 61% in 2006 (compared with the target of at least 70%) and the treatment success rate improved to 84.7% in 2005, just below the WHO Millennium target goal of 85% [111].

However, a recently published Cochrane review of direct observed treatment (DOT) – the third component of the DOTS strategy - aimed to compare DOT with self administration of treatment [112]. Eleven trials with 5609 participants were included. No statistically significant difference was detected between DOT and self administration, neither with cure nor completion of treatment as endpoints. DOT provided at home compared with DOT provided at a clinic suggested a possible small advantage with home-based DOT. There was no significant difference in success rates between DOT supervised at a clinic versus by a family member or Health Care Worker (HCW), or for DOT provided by a family member versus a HCW. In conclusion, the review of randomized controlled trials conducted in low-, middle-, and high-income countries provided no support for DOT. In comparisonwith self administration DOT had no significant effect on cure or treatment completion in people receiving treatment for TB. They concluded that DOT was unnecessary and disrespectful of patients [112].

Obermeyer et al. performed an empirical evaluation of the DOTS strategy, and found that DOTS had no statistically significant impact on case detection in a wide range of models and specifications. Howver, DOTS population coverage had a significant effect on overall treatment success rates in such a way that countries with full DOTS coverage benefit from at least an 18% increase in treatment success [113]. One of the main reasons for this seems to be geographical access for all to TB treatment in DOTS covered areas.

In their recent review of DOTS, Cox et al. found that the implementation of DOTS undoubtedly has improved the outcomes for millions of patients. However, they conclude that DOTS can only produce good outcomes reliably under "ideal" conditions, and can be of limited use in high burden settings, where challenges for the provision of even basic health services are manifold and complex [114].

In defence of DOTS, Davies and Squire state that it is too soon to conclude on the efficacy of DOTS for treating TB [115]. WHO has also vigorously challenged reluctant attitudes towards DOTS. It is argued that direct observation requires strong leadership and a lengthy commitment of human resources, which is often not yet the reality because of inadequate TB programs. The DOTS supporters have also launched principal arguments: it is believed that the ultimate ethical and legal responsibility for ensuring treatment completion and cure of a communicable disease belongs to the public health system and the community, and not to the individual patient [116].

Recent studies from Norway and Sweden also supports the use of DOTS. Norway and Sweden are comparable societies except that Norway fully complies to the DOTS strategy, whereas Sweden does not. Transmission of TB is stable in Norway, despite the import of TB through immigration and an increasing TB incidence. Immigrants from regions with high rates of TB do not significantly contribute to the spread of disease in the resident population [117]. Serious shortcomings have been revealed in the Swedish TB control program, including massive spread of drug resistant *M. tuberculosis* in Stockholm [118]. The epidemiology of TB in the two countries is different. It seems likely that by introducing obligatory DOTS to all patients, Norwegian health personnel accomplish treatment in an increasingly diverse population. In Sweden, however, control is complicated by the lack of DOTS. This situation has been promoted as a strong argument for introducing DOTS in all countries where it has not yet been implemented [119].

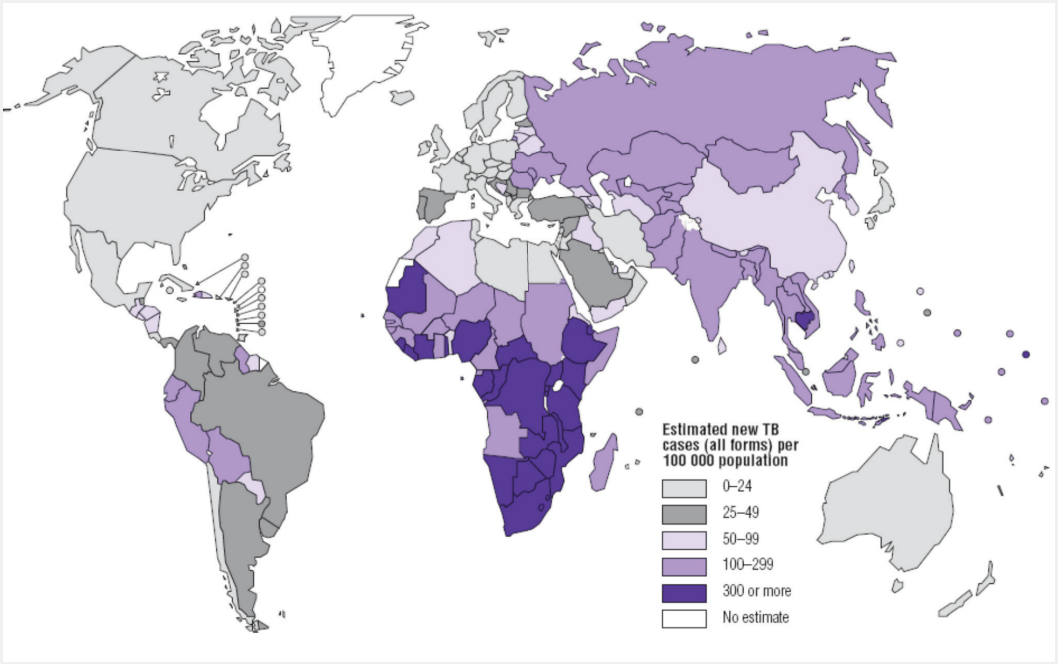
1.5.2 Drug resistance

Rapid and accurate diagnosis of symptomatic TB is critical for the control of TB. The resurgence of TB worldwide has been accompanied by an increase in the incidence of MDR TB on all continents [120]. The introduction of efficient anti-TB drugs was inevitably followed by development of drug resistance. In *M. tuberculosis*, drug resistance does not occur by acquisition of new resistance genes, as described for many other bacteria, but by random mutations in chromosomal genes. These mutations are spontaneous and do not depend on exposure to drugs [121]. For most drugs, the product of multiple genes constitutes the drug target and thus the sites for mutations leading to resistance are plenary [122]. The striking exception in this situation is that RMP resistance is mediated by mutations in the single gene encoding the beta subunit of RNA polymerase (*rpoB*) only [123]. The development of resistance to a drug

in the bacillary population follows from positive selection of a very small number of spontaneously mutated individual cells. Thus, the development of multiple resistance is due to a “domino effect” of periods of practical mono- or oligo-therapy [124]. TB control has a very limited number of effective anti-TB drugs at hand. It soon became obvious that if bacilli developed resistance to both RMP and INH, termed multi-drug resistance (MDR), successful treatment is difficult [100]. The treatment results have not improved much over the years despite heavy investments in second line drugs. Since RMP is the cornerstone among the first line drugs and MDR often follows once this drug fails, the logical thing to do is to define RMP resistance as the condition needed to be addressed [125].

MDR-TB poses a most serious threat to TB control; however, the experience in some places has been more promising than expected. Some countries have managed to prevent MDR in their TB population through strict drug policy [126]. But once MDR is established in the population, it is hard to curb it. The reason for this seems to rest partly in the DOTS strategy itself. When TB patients are brought closely and repeatedly together for diagnosis and administration of anti-TB drugs, they are exposed to cross- and super-infections from other TB patents. That this is a hazardous routine is also understandable from the fact that these patients share a number of risk factors and it has recently been shown that cross- and super-infections indeed occur [49, 127]. In South Africa Warren et al. found that 57% of patients infected with a BL strain were also infected with a non-BL strain. These results suggest that multiple infections and reinfections are frequent in South Africa, and that the initial infection does not induce an efficient protective immunity [49]. Strains that already have acquired drug resistance will be increasingly selected and cross-infections may become an additional problem to the lack of compliance that may cause relapse and treatment failure. It is therefore of the utmost importance to introduce evidence based treatment at geographic and national level, and to develop a rapid test for RMP resistance to be performed at the first encounter between the patient and the specialized TB services to prevent amplification of the MDR problem within health institutions [127].

Estimated TB incidence rates, by country, 2006



1.6 EPIDEMIOLOGY AND RISK FACTORS FOR TB

1.6.1 Epidemiology of TB

During the 20th century a steady decrease in TB incidence took place in the industrialised parts of the world. This started even before the introduction of mass BCG immunisation, anti-TB chemotherapy, and active screening programs based on TST and Chest X-ray. The decline was strongly associated with improved socio-economic conditions, better nutrition and enhanced housing conditions. It is assumed that more indoor space directly decreased the possibilities for transmission. Other factors such as pasteurization of milk, improved health services and understanding of communicable diseases, introduction of diagnostic tests and use of sanatoriums may also have contributed [128]. In the 1980s, however, the incidence of TB in these areas increased again. At the same time a doubling or tripling of notified TB cases was also seen in developing countries. In the industrialized countries factors as immigration from high-burden countries, the HIV epidemic and later in the 1990s the breakdown of the health infrastructure in the former communist countries have been pointed out as contributing factors [8]. For the high-burden countries increasing life expectancy,

population density, urbanization and extreme poverty as well as the rapidly spreading HIV epidemic and incomplete TB control programs apparently contribute to the increasing prevalence of TB [104].

Any factor that will prolong the period of infectivity of the TB patients will increase transmission rates of *M. tuberculosis*. From an epidemiological point of view, no treatment is favourable to an unsuccessful treatment where DR or MDR is acquired. As a result, the DOTS strategy emphasises on treatment outcome rather than on case detection. Enarson points at two factors that he considers responsible for an unsatisfactory outcome of treatment in a well-structured TB program. The first is diagnostic delay and poor adherence to the prescribed treatment regimen. The second is resistance to the medications used in the treatment [129]. These factors are discussed elsewhere in this thesis.

As mentioned previously, it may be that current successful strains represent an important “driving force” behind the current pandemic. In North-West Russia, Toungoussova et al. found that the above mentioned “classical” reasons for increased transmission could not explain the rising epidemic, whereas a rapid spread of MDR strains of the BL seemed to be the most likely explanation [8].

The next paragraphs outline the architecture of lineages of *M. tuberculosis* that have been described by molecular epidemiology.

1.6.2 Lineages of Mycobacterium tuberculosis

Utilizing Spoligotyping Sola et al. found 259 shared types among 3,319 isolates from 47 countries, and six major clades of tubercle bacilli [130]. Filliol et al. found nine superfamilies of strains: *M. africanum*, Beijing, *M. bovis*, East African-Indian (EAI), Central Asian (CAS), T group of families, Haarlem, X family, and Latin American-Mediterranean (LAM) family [131] (see also the chapter on TB evolution and aetiology above). The SpolDB4 presented by Brudey et al. defines 62 genetic lineages/sub lineages, also called clades/subclades (a c*lade* consists of all descendants of a common ancestor strain) [11].

1.6.2.1 The East African-Indian lineage

This lineage is characterized by a group of strains carrying a low number of IS*6110* copies and another group with high numbers of IS*6110*. EAI is frequent in South East Asia, India, and East Africa. It has been speculated that this lineage may have originated in Asia, where TB could have historically found favorable spreading conditions [132, 133]. Within the EAI lineage, new prototypic spoligotyping-signatures for 4 sub lineages were presented by Brudey et al. The EAI2 clade, previously named the "Manila family", was merged with a group of strains from Thailand. EAI3 and EAI4, were phylogeographically specific from India and Vietnam respectively, with suggested designations of EAI3-IND and EAI4-VNM. Two lineages from Bangladesh were designated as EAI6-Bangladesh/1 and EAI7-Bangladesh/2. EAI6-Bangladesh/1 harbours specificity for the eastern part of the South Asian region since it was also found in neighbouring Myanmar.

1.6.2.2 The Beijing lineage

In brief, Beijing and Beijing-like strains represent about 50% of the strains in some studies from Far East Asia and 13% of isolates globally [134]. It must be emphasized that these studies are largely performed in major cities and may not be representative for rural areas. The Beijing genotype belongs to the genetic group 1 of Sreevatsan, and has a specific spoligotype signature defined by Kremer et al. as absence of spacer 1-34, presence of at least three of spacers 35-43 and insertion of IS*6110* in the genomic *dnaA-dnaN* locus [58]. In the Beijing region of China, this particular genotype was found in >80% of the TB patients and was thus called the Beijing genotype [7]. It has been speculated that the BL is a group of variant clones that evolved from a common precursor maybe during the Genghis Khan reign or before [135]. It should be emphasized that the BL is a highly diverse group of strains and that the virulence of various isolates vary widely.

The variable number tandem-repeat (VNTR) typing system (see 1.3.3.2) is currently being utilized to define sub-groups within the BL, particularly the MIRU 26, VNTR 1982, and VNTR 3232 loci seem to have a high discrimination power [136]. A study in North West Russia found 96% of BL strains to be "W strain/typical", while 4% were classified as "atypical", because they were more distant from the rest. Typical and atypical Beijing strains may be discriminated on the basis of specific IS*6110* inser-

tion sites [6]. The typical strains are likely to be of monophyletic origin and their dissemination has started recently. The atypical BL strains are evolutionary “older”; they probably have a common ancestor with typical BL strains [137].

1.6.2.3 The Central Asian (CAS) family

Utilizing IS*6110* RFLP and spoligotyping the presence of a specific lineage that has been named the Central Asian (CAS) lineage has been reported from the Indian sub-continent [4, 131], Sudan, other sub-Saharan countries and Pakistan [11]. By IS*6110* RFLP the CAS lineage shows a band pair in the high molecular weight region (12.1 and 10.1 kilobase pairs) in addition to multiple other copies. The characteristic spoligotype signature is represented by the absence of spacers 4-27 and 23-34 [11]. Brudey et al. demonstrated 22 lineages/sub lineages of *M. tuberculosis.* They split the CAS lineage into CAS1-Dehli type and CAS1-Kilimanjaro [11]. Later other sublineages of CAS have been described [138]. It has been demonstrated that the CAS family has been particularly successful in East Africa. The low diversity of the highly prevalent CAS family in Tanzania indicates that this family is spreading rapidly or reflects a slower evolution of the DR region which could possibly be a result of the missing spacers in the central part of the spoligopatterns of these strains. The CAS- and EAI-families which were found to be abundant in Tanzania have previously been identified to have the most ancestral roots [40]. The absence of the Beijing family in East Africa indicates that it is unlikely that import of strains from Asia had a major impact on the *M. tuberculosis* population in Tanzania. This suggests an African origin of CAS and possibly of *M. tuberculosis* in general.

1.6.2.4 The Haarlem family

The Haarlem family was first described in the Netherlands by Kremer et al. [5]. It is characterized in spoligotyping by the absence of spacers 29–31 and 33–36. With IS*6110* RFLP these strains have a double band at 1.4 kb [139]. Within the Haarlem lineage, 4 sub lineages have been described. It has been suggested that Haarlem-4 strains represent an intermediate genetic link between the Haarlem-1 and Haarlem-3 genotypes [11]. More than 60% of the detected Haarlem strains have been localized in Armenia, Austria, Finland, Georgia, Iran, and Russia. The Haarlem family is also highly prevalent in Northern Europe, and to a lesser extent in the Caribbean. It is also

found in Central Africa and Saudi-Arabia, possibly introduced by European colonization and migratory activities [140].

1.6.2.5 The Latin American and Mediterranean (LAM) family

.The LAM family was originally defined by the absence of spacers 21-24 in the spoligotyping and the presence of an exact tandem repeat (A) allele equal to spacer 2 [130]. The diversity within the group is substantial, and according to the SpolDB4 12 subtypes have been defined [11]. Within the LAM lineage, the LAM7 sub lineage was renamed LAM7-Turkey. Similarly, the LAM10 sub lineage was renamed as LAM10-Cameroun. Recently, two new sub lineages have also been presented; LAM11-ZWE and LAM12-Madrid1. The LAM clade is frequent in Mediterranean countries and in Latin America, but is increasingly found also in Africa and other countries. LAM is well-known for the XDR-TB outbreak in South-Africa [141], that killed 52/53 patients and received intense international attention. Paleopathological data support the pre-existence of TB before the arrival of the Spanish conquistadors; it may have been endemic in Latin America or Africa or both, later spreading to Europe. But the LAM strains might as well have been introduced by the Europeans [142].

1.6.2.6 The X family

The X family (X1-X3 sub lineages) of strains is defined by a low number of IS*6110* copies and the absence of spacer 18 in the spoligotyping [143]. This genotype is today a well-characterized family. It is prevalent in UK, in USA and in former British colonies. The CDC1551 strain belongs to the X family, which was once suggested to be highly virulent. The X family seems to be linked to Anglo-Saxon countries. It was the first group identified in Guadeloupe and French Polynesia and is also present in South Africa, the Caribbean, Mexico and India [131].

1.6.2.7 The T family

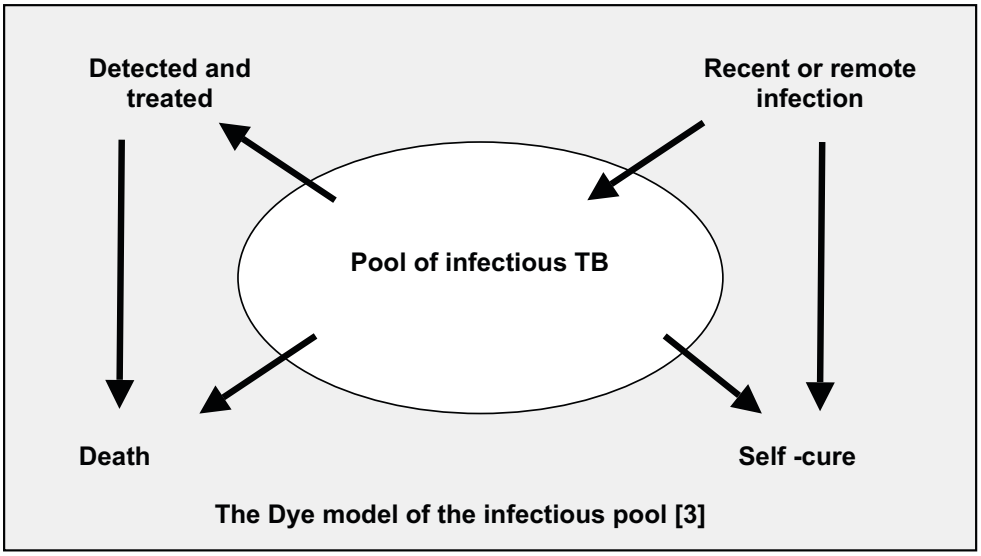
The "T" family still remains ill-defined with more than 600 unclassified spoligopatterns. They were stratified into 5 subclades (T1-T5) based on single-spacer differences by Brudey et al. [11]. The T family belongs to the PGG 2 and 3, are loosely

defined by the absence of spacers 33-36 in spoligotyping, and have been grouped together because they are difficult to link to other families [40]. If intact polyketide synthase genes are present, they appear to be more virulent than other strains [144]. A total of 8 nested clades, with robust spoligotyping-signatures were extracted from the 5 sub-clades. With the exception of "Tuscany", their names indicate their proximate upper-clade designation (T1 to T5), followed by their presumed geographical specificity: T3-Ethiopia, T5-Russia/1, T1-Russia/2, T3-Osaka, T5-Madrid/2, T4-Central Europe/1, T2-Uganda, and "Tuscany". The strain that caused the large outbreak of drug resistant TB in Stockholm in the years 2003-2005 ws also assigned to this lineage [118].

1.7 THE POOL OF INFECTION

1.7.1 Diagnostic Delay is a key variable to monitor the pool of infectious TB at a local level

A goal of TB control programs is to prevent transmission within the community. Achieving this goal takes considerable time, because most individuals in endemic areas are already infected, constituting a reservoir that continuously contributes to the pool of infectious cases. An effective TB control program requires early diagnosis and immediate initiation of treatment. Delay in diagnosis is important for disease prognosis



The Dye model of the infectious pool [3]

at the individual level, transmission within the community and the reproductive rate of the TB epidemic [3, 145]. Most transmissions occur between the appearance of cough and initiation of treatment. Madebo et al. found that patients become more contagious as the delay progresses; the longest delays are associated with the highest bacillary numbers on sputum smears [146].

The main indicator used by the WHO is the case detection rate (CDR), which is defined as the notification rate of new cases of smear-positive (ss+) TB divided by the estimated incidence rate. The WHO has defined a CDR of 70% as a target under the Millennium Development Goals [147]. CDRs are calculated based on tuberculin surveys, but these surveys require resources and expertise that is often unavailable. An alternative and more feasible approach could be to use data that can be obtained from local TB programs' records, eliminating the need for additional resources.

Among the possible variables that could be utilized to monitor the pool of infectious TB in a population is diagnostic delay (DD). Several models have been developed to describe the infectious pool, and one of the most comprehensive is the dynamic model of Dye et al. (above figure). Individuals enter into the pool when they develop active pulmonary TB and become infectious at a certain rate, and leave the pool as they start treatment, self-cure or die at certain rates. The size of the pool, and hence the burden of contagious cases, is defined by the relative size of these rates [148]. Each patient's contribution to the infectious pool is equal to the number of days that he or she remains infectious, that is, the interval between the onset of symptoms and two weeks after the patient starts treatment, when he experiences self-cure, or dies. There is convincing evidence that a large majority of TB patients are able to recall the onset of symptoms with acceptable accuracy [149]. In our study we intended to perform a systematic recording of the diagnostic delay for all categories; new pulmonary TB cases, retreatment cases, failures and undiagnosed cases, and hence obtain an estimate of the total infectious pool. Of these four categories, new sputum spear-positive TB cases contributed the greatest number of infectious days. We hypothesized that a local TB program could use a systematic recording of diagnostic delay as a quantifiable variable to monitor the infectious pool (PAPERS III, IV).

1.8 THE NEED FOR NEW DIAGNOSTIC TESTS

1.8.1 High endemic areas: an urgent need for a feasible, inexpensive and highly sensitive screening test that discriminates active from latent TB

The diagnosis of active pulmonary TB in high endemic countries is primarily based on microscopy of sputum-smears, which are neither sufficiently simple, rapid nor sensitive to serve as a feasible screening test. Sensitivity is low, because more than 10^4 bacilli per ml of sputum are required for detection, and approximately 50% of the active pulmonary TB is smear negative [117]. Lack of efficient diagnostic tests for sputum-smear negative (ss-) TB is among the major challenges combating the worldwide increasing TB epidemic.

Globally, there were an estimated 709 000 new HIV-positive TB cases in 2006 [111], and TB is killing 1 out of 3 subjects who die with AIDS [150]. In HIV-infected individuals delayed diagnosis of TB for more than three weeks after onset of active pulmonary TB is associated with 45-85% of the deaths [151]. More than 50% of the HIV cases with TB have a negative sputum-smear test [152].

More than 95% of patients with chronic cough do *not* have TB, and early contact with health services causes delay rather than suspicion of TB [153]. In Mumbai, a typical high endemic urban area, 80% of healthy individuals were found to be latently infected with TB [154]. The major task of a new screening test is therefore to differentiate active pulmonary TB from latent infection and previous BCG vaccination. This because the strategy should be to refer the individuals who come out as positive with the screening test to the National TB Program for further investigation with sputum microscopy and a clinical follow up system. The tests do not need to have a particular high specificity, but the sensitivity needs to be as close to 100% as possible.

Molecular techniques such as genome sequencing, cloning and expression, and purification of proteins have identified novel antigens for serodiagnosis of TB. ELISA-based serological tests utilizing a range of different antigens have been introduced, unfortunately all with low sensitivity and specificity [155]. This is mainly because of the heterogeneity of the antibody response in TB patients from different geographical locations. Antigen recognition in infected individuals is also highly heterogeneous due to factors such as stage of disease, strain of the bacilli, bacillary load, difference in human lymphocyte antigens (HLA) types and competence of the immune

system. In addition, in the human host there is a substantial cross-reactivity between *M. tuberculosis* and environmental mycobacteria, BCG and other bacteria such as e.g. *Nocardia asteroides* and *Corynebacterium pyogenes* [156]. This implicates that the antibody specificity is depending on the microbiological environment the individual has been exposed to before she is exposed to *M. tuberculosis* [157].

Due to advances in molecular biological techniques, an increasing number of novel antigens are being identified. The most promising antigens have been fusion proteins like CFP-10, *M. tuberculosis 8, M. tuberculosis 48, M. tuberculosis* 81 and the 38-kDa protein, which show encouraging results in detecting antibodies in sera of patients, also in sera of those with TB-HIV co-infection.

In a review of *M. tuberculosis* antigens with serodiagnostic potential Abebe et al. divided the candidates into six categories based on the type of TB they preferentially detect [155]:

1. Antigens that can be used to detect latently infected patients or household contacts:
16-kDa (a-crystallin), 14-kDa and 6-kDa antigens.
2. Antigens that can be used to detect TB patients at an early stage of disease:
M. tuberculosis 81, MPT51, MPT32 and Ag85C.
3. Antigens that can be used to detect TB patients co-infected with HIV:
TB9.7, TB15.3, TB16.3, TB51, *M. tuberculosis* 81, MPT32 and Ag85B.
4. Antigens that can be used to detect antibodies in sera of patients with extrapulmonary TB:
DAT, TAT, SL-I, cord factor, GST-822, ESAT-6, *M. tuberculosis 11* (CFP-10), TB9.7, TB15.3, TB16.3, TB51 and 38-kDa antigen. IgA antibodies to MPT64 have been studied in pleural effusions. MPT64 is also readily detected in tissues of TB patients using immunohistochemistry.
5. Antigens that can be used to detect patients with active pulmonary TB:
ESAT-6, *M. tuberculosis 11* (10-kDa), 19-kDa, 14-kDa, 16-kDa, MPT64, *M. tuberculosis 48, M. tuberculosis 81* (88-kDa), MPT51 (27-kDa), A-60, TB9.7 (10-kDa), TB15.3 (15-kDa), TB16.3 (16-kDa) and TB51 (51-kDa).
6. A combination of recombinant proteins and a fusion polyprotein which may detect different forms of TB (caused by different strains and in different host populations):
the 38-kDa antigen, *M. tuberculosis 8, M. tuberculosis 11, M. tuberculosis 48*, *M. tuberculosis 81*, DPEP, TB9.7 and TB16.3.

A particularly interesting study conducted by Mukherjee et al. in Calcutta focuses on peptides encoded by the RD1 region Rv3872. The immunological reactivity against a mixture of two peptides (P8 and P9) encompassing amino-acids 57–84 correlated well with that obtained using full-length Rv3872. They showed the highest level of antibody response in comparison with other peptides. Most interestingly Mukherjee's research showed that a mixture of P8 and P9 was able to discriminate between pulmonary TB patients and healthy BCG-vaccinated individuals [158].

1.8.2 Low endemic areas: The need for effective screening tools that can be directed towards risk groups

TB used to be among the most serious public health threats in Norway. During the last fifty years this has changed radically, as the new case detection has decreased to only 294 cases in a population of 4.5 million in 2007, out of which approximately 80% where born outside Norway. New immigration, increasing internationalization and reactivation can change this situation although the control program in Norway can still handle the challenge of imported TB [117]. In a recent 12-year study, importation of *M. tuberculosis* was shown to lead to increased incidence of TB but otherwise it had little effect on the TB situation in Norway. The social situation of the immigrant population was expected to have changed during this 12-year period. These changes were in part used to explain the changing TB epidemiology, but the low number of clustered strains could not support the statement that public health was hampered by immigration from high-incidence countries [117].

To prevent a resurge of *M. tuberculosis* in Norway the Government has given TB a high priority. One of the results is the new law on TB control, where screening of risk groups as immigrants and health personnel as well as obligatory DOT and effective contact tracing are key issues [159].

Compulsory screening for TB in the form of chest X-ray and tuberculin skin tests (TST) on entry has been implemented in Norway since the mid-1970s for individuals from high TB prevalence countries who intended to stay more than three months [159]. In a retrospective cohort study of 19912 asylum seekers with a mean follow-up of 6.3 years 76 persons were diagnosed with TB, of whom 43 (57%) had pulmonary TB. Twenty-two cases were found through screening at entry. Eleven persons had abnormal mass miniature X-rays, but had inadequate follow-up and were

diagnosed later through passive case finding. Mass miniature X-ray itself had a sensitivity of 96% and a specificity of 98% for pulmonary TB, diagnosed within 1 year after arrival [160].

Delay in start of TB treatment causes patients to have more advanced disease, more complications, higher mortality and more TB transmissions from each case [161]. Styblo calculated that a patient with untreated ss+ pulmonary TB may infect on average more than 10 patients annually and over 20 during the natural course of untreated disease until death [101].

Diagnosing latent TB has been based on TST. This test has many disadvantages, but has been used due to the lack of better alternatives. The main problem of the TST is the low specificity [162]. Both BCG immunization and exposure to environmental mycobacteria can result in a false positive TST. In addition, 15%-25% of those with active TB have a negative TST [163]. The sensitivity of the TST is easier to define than their specificity in surveys. Given a normal distribution, the number of infected individuals missed because they have an induration less than the cut-off value can be calculated. For culture confirmed TB there are diverging reports of sensitivity from 100 % (McConkey et al. [164]), 89 % (Britton et al. [72]), 69 % (Lalvani et al. [165]) to 63 % (Liebeschuetz et al. [166]).

Among the risk groups we targeted health care workers (HCW) at Norwegian hospitals (Paper V). The current policy is to perform environmental screenings after cases of unprotected exposure to ss+ pulmonary TB if a case of not priory known pulmonary TB was admitted, and no isolation or preventive measures were established. In concordance with the national guidelines super-infection is defined as an increase in TST induration from the last previous TST of ≥ 10 mm or a TST of ≥ 15 mm if previous TST status is unknown [159]. The individuals defined as super-infected are followed up with consultations at the pulmonary outpatient department and annual chest x-rays for three years.

However, recently developed in vitro assays based on T cell production of INF-γ in response to the *M. tuberculosis* specific antigens ESAT-6 and CFP10 have contributed to improved specificity in TB screening. These RD1 encoded protein antigens are absent in all vaccine strains of *M. bovis* BCG and most non-tuberculous mycobacteria (except *M. marinum, M. szulgai, and M. kansasii*), and such tests can therefore distinguish *M. tuberculosis* infection from infections caused by other mycobacteria or previous BCG-vaccination [165, 167]. Two commercially available and

regulatory agency approved (EU, Quantiferon-TB Gold (QFT) (Cellestis) also by FDA). Two test systems can be used: T-SPOT.*TB* (Oxford Immunotec) is an ELISPOT assay based on analysis of a defined number of isolated blood cells, whereas QFT is a whole blood ELISA based test. The tests have comparable specificity (98-99 %), but sensitivity is reported to be somewhat higher for the T-SPOT.TB test (97 %) as compared to QFT (90 %). This difference is most pronounced in immuno-suppressed persons and in children, where the frequency of indeterminate results is demonstrated to be higher for the QFT test [168, 169].

With their high specificity, IFN-γ release assays have the potential to improve both the diagnosis of TB in infected individuals as well as the utilisation of public health resources for TB control. The tests are not suitable for high endemic areas because they are not able to differentiate between latent infection and active disease, and they are too resource demanding for poor countries. In paper V we compared the T-SPOT.*TB* test with TST in TB exposed hospital personnel and used the results to assess the role of IFN-γ release assays for improved screening of this target group as well as the rate of TB transmission during short-time exposure in a low TB endemic country.

1.9 TO OVERCOME CULTURAL, SOCIOLOGICAL AND ECONOMICAL CONSTRAINTS TO AN EFFECTIVE TB CONTROL PROGRAM AT A LOCAL LEVEL. THE DECENTRALIZED BRAC MODEL AS AN EXAMPLE.

A panel of Health Care Workers in KwaZulu-Natal defined stigma, denial, and lack of education as the primary reasons for failure to complete TB treatment. The results also highlighted inadequate support for and monitoring of the program as a result of a lack of administrative supervision, which is typical in many high-endemic countries with weak health care infrastructures [170]. A similar qualitative study from Swaziland raised two main societal issues, which had major impact on the success of the TB program: health beliefs and poverty. It is seen that health beliefs can have a major impact on treatment-seeking behaviour and outcomes of TB treatment. Problems related to poverty were of two main types: insufficient funds to attend for review, and lack of food whilst on TB treatment [171]. There is no doubt that TB in many aspects is “the poor man’s disease”. In Brazil a recent study found the socioeconomic factors to

account for 87% of the total variation of the disease prevalence. The disease prevalence was clearly higher in the poorest areas [172].

In our implementation of the field work in Bangladesh (Paper I, II), we co-operated with Bangladesh Rural Advancement Committee (BRAC), the largest NGO in the world, employing approx. 100 000 people. The twin objectives of BRAC are poverty alleviation and empowerment of the poor, and to provide and protect livelihoods of around 100 million people in Bangladesh. The organisation implements programs on poverty alleviation through micro financing, non-formal primary education, women's health measures, and disease control [173].

BRAC has been implementing a TB program in Bangladesh with DOTS since 1984, well before the DOTS strategy was launched by WHO in 1997. Since the implementation of DOTS, consistent success rates of around 85% have been reported. In the BRAC program, the responsible community health worker (CHW), the so called *Shaisto Shebika,* comes from the village in which she works, and thus knowledge about the family situation and culturally appropriate interaction with patients is ensured.

The TB-control program, which is implemented in collaboration with the Government of Bangladesh (GoB), has gradually been scaled up to cover a population of 83 million [173]. At the core of the program is the *Shaisto Shebika*, the CHW, who is a member of the local village organisation and supported by BRAC through micro-financing and other development inputs. CHWs are all women, mostly unschooled, who receive training from BRAC on the treatment of common illnesses including TB. The CHW identifies people with chronic cough and sends samples of sputa to a local BRAC laboratory for microscopy. The ss+ cases are brought to treatment immediately. The CHW provides the drugs, which are received free from the GoB. During the initial 2 months after identification, the patients come to the CHW's home daily for drugs. For the remaining period of treatment, the patients are given a week's supply at a time. Before the CHWs initiate the treatment, the patient has to deposit a sum of money and sign a bond that she or he will complete the treatment; in case of default, the bond money is forfeited. The factors responsible for success include the learning culture of the BRAC organisation, involvement of CHWs, the bond money requirement, program out-reach, provision of free drugs, good management, and availability of technical and financial resources.

A second issue raised by the Bangladesh experience is the need to demystify TB treatment. The success of the CHW approach challenges the myth that physicians

are essential at all stages of TB treatment. Trained local women with good supervision can accomplish seeming miracles in the villages of developing countries. The more TB treatment is brought outside hospital walls and the dependence of experts, the greater the success of DOTS; the example of oral rehydration therapy in many developing countries including Bangladesh bears testimony of this.

1.10 TB IN BANGLADESH

Bangladesh has the sixth highest burden of TB cases among all nations. For 2006 it was estimated that there were 300,000 new cases, out of which 137,000 were infectious, ss+ cases. The annual incidence of TB was 101 per 100,000 population for ss+ cases and 225 per 100,000 for all forms. It was estimated that TB caused 70,000 deaths. The official case detection rate was as low as 41%, but due to a substantial private health sector and that TB drugs are available on the open marked the correct CDR is difficult to estimate. The success rate of detected cases under DOTS was 84%. With an estimate of 300,000 new cases annually, those cured under the NTP comprised only 3.2% of all new cases in 2003 [104].

A TB service delivery survey performed in 2004 found multiple reasons behind the low case detection rates. First, too few diagnostic centres where sputum microscopy is performed. One microscopy centre per 100,000 population is recommended in high-burden countries, but a 2004 WHO investigation indicated that in Bangladesh each *Upazilla* (sub-districts of approximately 300,000) is served by only one centre with a sputum microscopy facility. Second, only about half the population had real access to DOTS services. In most urban areas, the district hospitals, academic institutions, private clinics, and institutions like prisons and corporate hospitals had not implemented DOTS. About 70% of the microscopy centres in the DOTS program delivered substandard services, both due to poor training and availability of refreshment courses and follow up of the laboratory technicians [174]. An estimated 60–70% of TB patients in urban areas seek care from the private sector. No records of patient numbers or treatment patterns are maintained within this service. The NTP assume that just over half of all patients seek care from chest specialists, health centres, and NGO clinics. Most of these providers do not offer DOTS supported treatment, although some of the NGOs have implemented DOTS [175].

Several other factors are also believed to contribute to Bangladesh's TB burden. After introducing DOTS in the NTP, there has been concern about the availability

of anti-TB drugs for the poorest patients has been compromised. First, it requires recourses to go to the clinic on a daily basis, resulting in a lack of income. Second, lack of education makes it also difficult for the poor to achieve the knowledge and understanding they need to be motivated for the completion of treatment. A study performed in 1991 in the rural area of Chagalnaya found an increasing prevalence with increasing age, a higher prevalence among males and among occupation groups like farmers, workers and housewives. In addition, TB was more prevalent among illiterates and those living in overcrowded, low standard houses. They also found that 85.7% of the detected ss+ infectious cases were formerly undetected and untreated [176]. Other studies have also detected high incidences in densely populated urban areas with poor living conditions [177]. Seventy-nine percent of new infectious TB cases are found in the economically active age groups (15-54) [177]. Gender also seems to be important; there are twice as many new ss+ cases registered for treatment among men than among women. A prevalence study confirmed the higher prevalence among men was real, and not due to female cultural constraints to achieve treatment [178].

1.10.1 TB treatment in Bangladesh

The Bangladesh NTP was officially established in 1993 through endorsement of the DOTS strategy for TB control (World Health Assembly, Geneva, May 1991). The NTP now covers 99% of Bangladesh's geographical area. Under the NTP, DOTS services are delivered from more than 500 Government-run centres and NGO clinics in all *upazillas*, districts, and city corporations [179]. There are two types of GoB facilities:

Upazilla health complexes (UHCs): There are 460 UHCs, each covering an average population of 270,000–300,000; UHCs are 31-bed hospitals with indoor facilities and field functionaries to deliver primary healthcare (PHC) services to the rural populations. UHCs are comprehensive sub-district level facilities that provide TB care along with a wide range of other preventive and curative services, including both in- and out-patient care.

Chest disease clinics (CDCs): The 45 CDCs are secondary-level care facilities in 44 district headquarters; they serve large and widely variating populations. They are devoted specifically to the screening, diagnosis, and treatment of TB patients. They serve as DOTS centres and support the NTP through diagnosis of pulmonary TB cases, proper referral of patients for treatment to respective UHCs, and provision of technical advice according to national guidelines. They refer complicated cases to ter-

tiary-level hospitals. There are also 11 major NGOs (with sub-awardees), including BRAC, that provide DOTS under the NTP umbrella. The NGO Service Delivery Program (NSDP) is one of them and is responsible for providing DOTS in the major urban areas, i.e. the city corporations. This is implemented through NGO clinics under the NSDP network. Not all NGO clinics in Bangladesh participate in the network of the NSDP. Although NSDP has both urban and rural NGO clinics, only some of the NGO clinics located in the city corporation areas provide DOTS support, in terms of diagnosis and care. Acid-fast bacilli microscopy is provided free in all DOTS centers, while chest X-ray is used for the diagnosis of smear-negative or complicated cases, mainly in the CDCs. TB-DOTS treatment is provided free from all centers and, as much as possible, decentralized at the community level through community health workers, village doctors, the GoB, and NGO staff [179].

1.10.2 Drug resistance in Bangladesh

The national drug resistance testing (DTS) facility operated by the NTP was discontinued in 1995. The 1996 WHO Report states that before the introduction of the NTP, and DOTS in 1993, up to 60% of the TB cases in Bangladesh were treated by private clinicians. Monotherapy and other inadequate treatment regimens were common, and anti TB drugs were available on the open market [180]. Since the early 1990s DOTS has gradually expanded to cover all regions of Bangladesh, but in 2003 the CDR of sputum ss+ cases was still only 33 %, compared to the WHO goal of above 70% [181]. In 1995 a baseline study at the start of the NTP was performed by sampling of pre-treatment sputum from all newly registered ss+ cases in the Greater Mymensingh District. In this mainly rural population resistance among new cases to INH was 5,4%, to RMP 0,5%. Resistance to INH and RMP in previously treated cases were 25,9% and 7,4% respectively. MDR-TB was observed in one new case (0,23%) and in 5,6% of previously treated patients. It was concluded that NTP regimens for new and retreatment smear positive cases are appropriate, the more so since HIV was virtually absent. Indications for the retreatment regimen should be extended to involve all patients who had any previous treatment for at least one month. The prevalence of drug resistance was found to be low, but may be expected to rise with improved economical conditions if the private distribution of TB drugs cannot be avoided [182].

Another study from 2000 in an urban TB clinic in Dhaka showed resistance in 29,7% of new patients to at least one drug; 15,8% to INH, 10,9% to RMP, 6,9% to SM, 2,9% to EMB and 3,9% to PZA. MDR-TB was found in 4,95% of the cases. Ciprofloxacin was tested against 30 strains of *M. tuberculosis,* 67% of which were resistant [183].

In Matlab, a rural area, also studied after the introduction of DOTS, MDR-TB was absent among new cases, while it was common (27.3%) among previously treated cases [184]. Studies from rural India (1997-2003) have demonstrated that resistance to RMP in new cases was 0.5-4.4% and that the prevalence of MDR-TB was 0.5-3% [185]. In conclusion TB drug resistance seems to be higher among new patients in urban areas than in rural areas, and substantially higher in previously treated cases than in new cases, both rural and urban areas. The same range of frequencies of MDR-TB among new cases were described in Myanmar (4.2%) [186], Thailand (4.2%) [187] and China (4.5%) [188].

Questions that need to be answered are: Why is the prevalence of TB in Bangladesh one of the highest in the world and why is it increasing? The prevalence of TB in Chagalnaya in 1991 was significantly higher than the rates found in the TB Survey of Bangladesh 1964-66 [189] and in the Epidemiological study of 1984 [190]. Seventy-nine percent of new infectious TB cases are found in the economically active age groups (15-54) [177]. Therefore, TB is not only a major killer but also a heavy economic burden on one of the poorest countries in the world. A major question arises: What kind of strains are contributing to the TB epidemic in Bangladesh? Do we face a situation of increasing dominance of particular emerging successful strains?

1.11 STUDIES OF MOLECULAR EPIDEMIOLOGY OF M. TUBERCULOSIS IN BANGLADESH

The information on the molecular epidemiology of *M. tuberculosis* strains in Bangladesh is limited. A study performed in 1999 included 48 strains from a hospital in Dhaka city. A total of 34 strains (71%) were grouped by spoligotyping into nine different clusters; the largest comprised 15 (32%) isolates of the BL, whereas the remaining eight clusters consisted of two to five isolates [4]. This material indicated that BL play a major role, but needed confirmation, especially by materials from rural areas like Su-

namganj. Also the study from patients in one hospital in Dhaka could represent an co-incidental outbreak, thus confirmation and expansion of population size was called for.

A study utilizing spoligotyping of non-hospitalized TB patients of both urban (Dhaka) and rural (Matlab) areas of Bangladesh were performed in 2001-2003 by Rahim et al. Spoligotype patterns were compared with the SpolDB4 database. A total of 193 of 224 (86%) isolates were clustered into 31 shared types (ST) of 2 to 34 strains, whereas 31 strains (14.0%) were unique. After matching with the SpolDB4 database the strains were clustered into four major clades: 75% percent of all strains belonged to the principal genetic group 1 (PGG 1) [191] that includes BL, EAI and CAS and 25.0% belonged to PGG 2 and 3.

EAI was the most prevalent clade and constituted 44% of the isolates. Most strains of the EAI clade could be classified into EAI1 to EAI5. Within the EAI clade, 49 strains had the spoligotype signature designated EAI6-Bangladesh1 (EAI6-BGD1). Other EAI subclades such as EAI4 (Vietnam) and EAI8 (Madagascar) were not found. The fact that "ancestral" TB clades [40] like the EAI are predominant in Bangladesh makes it likely that there has been a historical presence of endemic strains. Rahim argues that the emergence of an endemic new lineage (EAI6-BGD1) is also evident from the comparison with results from an unpublished study by Rigouts et al. (personal communication to Rahim). The EAI6-BGD1 lineage's origin in Asia is not yet precisely dated, but these strains belong to PGG 1 (assessed by *katG-gyrA* polymorphism [42]). On the other hand: a recent study documented a single nucleotide polymorphism located within the nitrate reductase (*narGHJ*) operon promoter clustering BL together with PGG 2 and 3 organisms [192]. The CAS family constituted 15.0% of the isolates in the study by Rahim et al. [191]. The predominant Prototype ST 26 is known as the Delhi type and is prevalent in north India [193]. Other present CAS 1 prototypes in the Rahim study were ST 794, ST1120, ST 288 and the newly described ST2149 [191]. This family is prevalent in Pakistan, Sudan, and Libya. The CAS 2 variant ST 288 was also detected. Modern strains (PGG 2 and 3) constituted 25.0% of the strains. The Latin-American and Mediterranean (LAM) clade was the second most prevalent modern clade. The ST 137, one of the European low IS*6110* copy clades, was also found [191]. It is related to T-PRT, known as the "Portuguese type" (ST 244) [11]. The Portuguese settled at the Indian subcontinent at the end of the nineteenth century, which confirmed the likely existence of a common ancestor of today's clones.

1.12 TB IN ETHIOPIA

TB is considered a major public health problem in Ethiopia, ranking as number seven just behind Bangladesh among the 22 TB high-burden countries [194]. In 2006 the incidence of new ss+ TB cases was 168 per 100 000 pop. The incidence of all new cases was 379 per 100 000 pop. and the total prevalence of all cases was 643. The annual TB death rate was 84 per 100 000 pop, or approximately 70 000 in a population of 81 million people [111]. In 2000 the worldwide percentage of MDR-TB was calculated to be 3.2% of all new TB cases, in Ethiopia it was 2.3%.

A study from Addis Abeba in 2004 found the incidence of MDR-TB in not previously treated TB cases to be 5,3%. They also found an increasing resistance to one ore more drugs from 14% in 1996 to 22% in 2001 [195].

Before the introduction of DOTS in Ethiopia, 82% of TB patients were reported to have failed to complete treatment [196], while defaulting after the introduction of DOTS has been reported as low as 11.3% [197]. In another study a significantly declining trend in treatment non-completion from 38% to 18% over six years during 1994–2000 was reported. Patients interrupted treatment mainly because of long distances to the health facilities, poor awareness about the disease and treatment length, side effects and lack of family support [198]. In Addis Abeba a qualitative study concluded that patients often discontinued treatment because of "social problems" and "feeling of improvement" [196].

Our field study in Bangladesh did not obtain information on diagnostic delay. The Amhara Region of Ethiopia was chosen to demonstrate the usefulness of DD as a tool to monitor the infectious pool of TB in a population because Yimer et al. had already performed a study in this area on DD in new ss+ patients [199]. By revisiting the area additional information from the NTP protocols was obtained on ss- patients, retreatment- and defaulted cases.

1.13 NORWAY

1.13.1 TB in Norway

Norway has one of the lowest incidences of TB in the world. Dahle et al. surveyed all notified TB cases in Norway between 1994 to 2005, and identified a total of 135 clusters, indicating a high genetic diversity. From 1994 to 2005, Norwegian annual TB incidence increased 11%, from 5.6 to 6.2 cases per 100,000. By 2005, foreign-born individuals (7.9% of the population) accounted for 78% of Norwegian TB cases - versus 46% in 1994. This striking finding reflects declining case numbers among Norwegian-born persons, a near-doubling of the foreign-born population, and increased incidence within this group [117].

The great majority of the cases are due to reactivation of previously acquired disease; the ethnic Norwegians are mostly elderly people who contracted the disease before 1960, and the foreign-born cases had a latent infection when arriving in Norway. Transmission between immigrants and native Norwegians is rare. Two major outbreaks have been identified; one mainly among immigrants and one among native Norwegians [200]. The fact that they had been going on for several years has also been described in other low endemic areas, indicating that even with a good national program for TB surveillance, outbreaks are difficult to identify mainly because TB is not suspected [201].

The key elements in TB control are to cure the individual patient, interrupt transmission of TB to others and prevent the tubercle bacilli from becoming drug resistant. Incomplete treatment may result in excretion of bacteria that may also acquire drug resistance and cause increased morbidity and mortality. Treatment outcome results serves as a tool to control the quality of TB treatment provided by the health care system.

A study conducted by Farah et al. evaluated the treatment outcome for new cases of culture positive pulmonary TB in Norway during the period 1996-2002. Among the 655 patients included, the total treatment success rate was 83%. Three percent defaulted treatment, 9% died and 4% transferred out. The default rate was higher among foreign-born and male patients [202]. TB in Norway is also discussed in section 1.8.2.

1.13.2 Norwegian Health personnel as a risk group

Several studies from the pre-fingerprinting era concluded that prolonged contact with an infectious case of TB was the usual transmission pattern. The risk of being infected could be calculated as a function of the time spent sharing room air with the index case [203, 204]. When contact tracing utilizing fingerprinting methods as *IS6110* RFLP and Spoligotyping became available, it was revealed that a substantial proportion of TB cases must have acquired TB infection as a result of a casual contact [205, 206].

HCWs are constantly at risk of unprotected exposure and a better understanding of *M. tuberculosis* transmission after brief exposure is essential to improve the follow-up routines. As most new infections result in latent TB infection, strain fingerprinting is not an amenable tool to trace transmission.The current policy in Norwegian health institutions is to perform environmental screenings based on TST after TB exposure.

A major problem in many Norwegian hospitals is that the same HCWs have undergone multiple screenings. Some wards like pulmonary and infectious disease units even routinely screen their personnel with TST annually. The result is a massive boostering, and it is not uncommon that more than half of the staff of these kinds of wards is defined as TST positive. Thus, due to the low specificity and utilization malpractices of the TST [167], a large proportion of the group defined as TST positive after exposure are probably false positives, which leads to incorrect treatment, waste of resources, and unnecessary anxiety. As discussed in section 1.8.2; two new blood tests, based on detection of INF-γ produced by T cells in response to antigens specific to *M. tuberculosis* and encoded by the RD1 region, have been found to be more accurate than the TST. Our study of exposed HCW in Norway (Paper V) compared TST to T-SPOT.TB.

2. MATERIALS AND METHODS

2.1 AIMS OF THE STUDIES

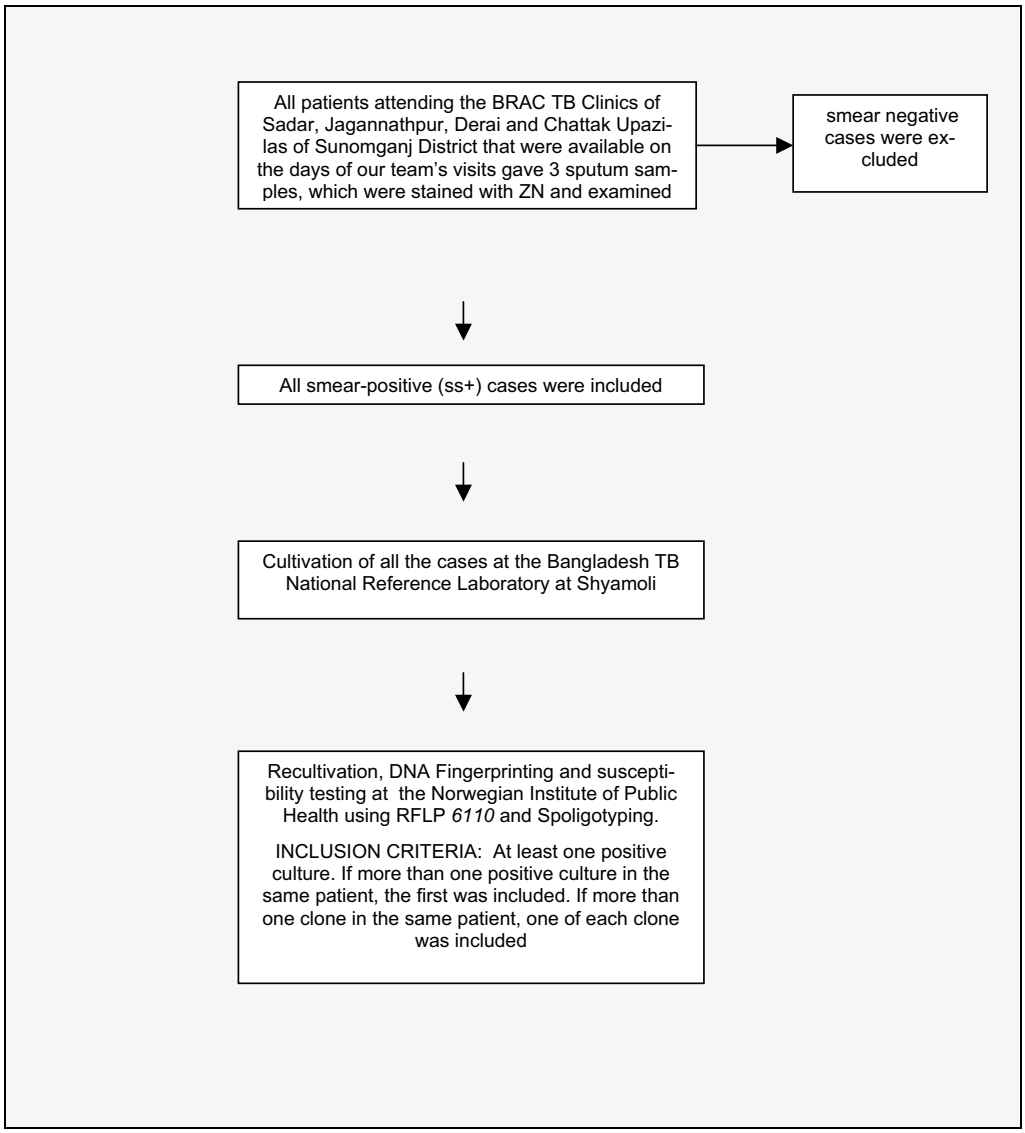
This thesis aimed at contributing to the current knowledge that forms the fundament for local, national and international control of TB.

SPECIFIC OBJECTIVES

1. MOLECULAR EPIDEMIOLOGY
    a. To describe the main families of *M. tuberculosis*, and detect the degree of reactivation in a rural area of Bangladesh.
    b. To describe the drug susceptibility pattern in isolates from ss+ patients in rural parts of Bangladesh.
    c. To characterise demographic, social and medical risk factors contributing to the spread of TB in rural parts of Bangladesh.
2. DIAGNOSTIC DELAY
    a. To evaluate if treatment delay can be utilized as key variable for monitoring the pool of infectious TB in a high-incidence region.
3. IDENTIFICATION OF RECENT INFECTION IN A LOW-ENDEMIC SETTING
    a. To determine the rate of *M. tuberculosis* infection after unprotected exposure of personnel in Norwegian hospitals.
    b. To compare the efficacy of the T-SPOT.*TB* test with TST as tools to detect infection in TB exposed hospital personnel.
    c. In the light of costs and benefits both at the individual and national level to evaluate improved follow up strategies for TB exposed hospital personnel.

2.2. BANGLADESH

2.2.1 Experimental design



2.2.2 Area and population at risk

Sunamganj district of the Sylhet division of Bangladesh is a typical rural district without any major urban areas located at the northeast border to India. According to the official statistics of the Bangladesh Bureau of Statistics for 1998 it had a rural population of 1.67 million and an urban population of 135 000. It consisted of 3670 sqkm, and with its 552 citizens per sqkm it was less densely populated than the average 855 for Bangladesh. The crude birth rate was 18.60, compared to 19.87 for Bangladesh. Especially the border areas used to be populated by a majority of indigenous people like Garo and Hajong, but recently there has been an immigration of Bengali speaking groups. The infrastructure is rather poor, and many villages are only accessible by boat. It has been considered a remote and underdeveloped area within Bangladesh [207]. According to the WHO, the 2003 annual risk of TB (ARTI) in Bangladesh was estimated to be 2,24%, which should give 40,364 new cases every year if Sunamganj is on the national average [208].

2.2.3 Sample size

The objective of the study was not only to test for drug resistance, but also to describe the epidemiology of the local *M. tuberculosis* population, and analyze the clustering patterns, with special emphasis on the spread of specific family strains. The project aimed to collect all strains isolated in the BRAC TB-clinics in the Sunamganj district for 1 year.

Due to difficulties related to transport and decentralised treatment clinics, members of the research team could only be present a small proportion of the time at each clinic. And adding loss of cultures during transport and cultivation in Dhaka, we expected the total number for strains to arrive the NIPH in Norway to be 200.

2.2.4 Collection of strains

BRAC, the largest national NGO runs a TB Program in co-operation with the Government in all the Upazillas of the Sunamganj district. BRAC diagnose and give free treatment to a majority of the TB patients. Following the guidelines of the NTP, three sputum samples are collected from all attending patients. Short-course chemo-

therapy (2HRZE/4H3R3) is given for pulmonary TB to sputum ss+ patients (infectious), under DOTS as close as possible to the community.

The study intended to include all the ss+ cases detected by BRAC in Sunamganj from November 2003 to December 2004, excluding the ss- and extra-pulmonary cases.

2.2.5 Cultivation and susceptibility testing of the strains

Four upazillas (sub-districts) in Sunamganj were included in the study. These were Chatak, Derai, Duarabazar and Sunamganj Sadar. The methods applied and the diversity of the *M. tuberculosis* population included in this study has been presented previously [209]. The isolates were collected from November 2003 to December 2004 (4 isolates were collected in early 2005). Due to the relative inaccessibility of the study area, sputa from 246 out of 743 ss+ cases diagnosed during the study period were refrigerated for approximately one week, during transport and prior to processing at the Centre for Health and Population Research (ICDDR,B). The samples were decontaminated following the standard procedure of Petroff [210], and inoculated on two Løwenstein-Jensen (L-J) slants for up to eight weeks. Out of the 246 sputa 148 isolates of *M. tuberculosis* were identified by the niacin accumulation- and nitrate reduction tests performed according to the WHO guidelines [211]. All mycobacterial isolates were transported to the National Reference Laboratory for Mycobacteria at NIPH in Norway, where they were re-cultivated and re-identified (Paper I). Identification was based on 16S-rRNA hybridization (AccuProbe; GenProbe Inc., San Diego, USA) or the niacin accumulation- and nitrate reduction tests [211]. Drug susceptibility testing (DST) was performed by the use of the Bactec MGIT 960 system (Becton Dickinson) following the standard user's manual. The anti-TB drugs tested and the concentrations used were as follows: INH, 0.2 μg/ml; RMP, 2.0 μg/ml; EMB, 7.5 μg/ml; streptomycin, 6.0 μg/ml. Multi drug resistance was defined as resistance to at least INH and RMP (Paper II). Cases previously treated for at least 1 month were defined as retreatment cases.

2.2.6 IS*6110* RFLP and spoligotyping

Fingerprinting with IS*6110* RFLP was started by harvesting of DNA from a bacterial culture. Unlike for PCR-based methods like spoligotyping, a relatively large quantity of DNA is required [212]. Thereafter DNA was digested with the restriction enzyme *PvuII* and the restriction fragments were separated by electrophoresis on an 0.8% agarose gel. The separated restriction fragments were transferred to a GeneScreene Pluss membrane (DuPont Boston, Mass.) by Southern Blotting. Hybridisation in order to visualize the IS*6110*-containing restriction fragments was done with a 245-bp digoxin-labelled PCR-amplified probe with a DNA sequence complementary to the IS*6110* DNA sequence.

Spoligotyping

Spoligotyping is based on the in vitro amplification of the DNA of the DR genomic locus present in the *M. tuberculosis* chromosome [57]. The reverse primer is biotinylated at the 5' end. A set of 43 oligonucleotides, each corresponding to one of the unique spacer DNA sequences within the DR locus, are covalently bound to a commercially available membrane. For hybridization, 20 µl of the PCR product is diluted and heat denatured. The samples are pipetted into parallel channels of a miniblotter in such a way that they are perpendicular to the rows of oligonucleotides. The hybridization is performed for 60 minutes at 60°C. The membranes are incubated with streptavidin-peroxidase conjugate for 60 minutes at 42°C. The patterns are visualized by use of the chemiluminescence and by exposure of the membrane to an X-ray film. The results are analyzed manually and by computer assistance.

Compared to other fingerprinting techniques spoligotyping requires less expensive equipment, so it can be utilized in laboratories with limited resources. It has also been demonstrated that the results are highly reproducible [213]. Tools like the SpolDB4 database [11] and the web-based computer algorithm 'Spotclust' searches [214] can be performed to assign isolates to specific families, subfamilies and variants. SpolDB4 is the largest and most up to date available global database for spoligotypes. Databases like SpolDB4 are important sources of knowledge of the world-wide spread of specific families of *M. tuberculosis.*

DNA isolation and IS*6110* RFLP were performed as previously described by van Embden et al. [212]. The Bionumerics software (Applied Maths, Belgium) was used to calculate the genetic relationship between the patterns and generate the dendrogram. The analyses were based on the Dice coefficient. A cluster was defined as two or more isolates from different patients with identical RFLP typing patterns. Spoligotyping was performed as described elsewhere and according to the manufacturers instructions [213].

Nieman et al. tested the stability of *M. tuberculosis* IS*6110* fingerprint patterns and spoligotypes by analyzing serial isolates from patients with drug-resistant TB and found that the IS*6110* fingerprint patterns of *M. tuberculosis* isolates have high degrees of stability. Compared to IS*6110*, the direct repeat (DR) region, which is the basis for spoligotyping, has a lower rate of change [215].

2.3 ETHIOPIA

2.3.1 Study group and design

In an attempt to operationalize the Dye model of the infectious pool [148], we systematically recorded treatment delay (TD) in a population of 18.1 million individuals in the Amhara Region of Ethiopia [216]. Each patient's contribution to the infectious pool is equal to the number of days that he or she remains infectious, that is, the interval between the day the patient can recall the onset of symptoms and the day the patient starts treatment. Retreatment cases remain infectious until they are cured or die.

By recording the treatment delay for new TB cases, retreatment cases and failures, and by estimating the number of undiagnosed cases, the total number of infectious days and hence an estimate of the infectious pool can be calculated.

A cross-sectional study was conducted between September 1, 2003 and December 31, 2003. The following zones were randomly selected from the eleven basically rural zones in Amhara as study sites: North and South Wollo, North Gonder, North Shewa, East Gojam and Bahir Dar. The study sites were all the five hospitals that are available in the selected zones. Besides this, we included 15 health care centers. We did not include any health stations because they do not have diagnostic facilities for TB. Participants were consecutively included and interviewed immediately after diagnosis until the intended sample size was achieved. The data was collected by nurses and health

officers who had been trained for the purpose. New ss+ pulmonary TB patients aged 15 or more years were included in the original study on TD [199], but all categories of pulmonary TB patients were interviewed, and the data for the other patient categories were retrieved for the purpose of this study at a later stage. Patient register card, TB registration books and laboratory registers were crosschecked to assure the data quality and the collection of data was closely monitored by the study supervisors. The sample size was calculated using the formula required for determination of sample size for estimating single proportions. By taking a previous study performed in Ethiopia on TD, which showed a 58% proportion of more than one month delay, a 95% CI and a margin error of 5% the sample size was calculated to 373, 384 patients were included. A pretested structured questionnaire was used to collect information on socio-demographics, the major presenting symptoms of pulmonary TB, the duration of the major presenting symptoms, and the date of the first healthcare visit. The major pulmonary symptoms included on the questionnaire were presence of cough for more than 3 weeks, production of sputum, chest pain, and hemoptysis. Patient register cards, TB registration books, laboratory registries and electronic data were cross-checked and validated to ensure the quality of data. The annual numbers for 2003 were interpolated. SPSS version 11.0 was used for analysis.

3.3 NORWAY

3.3.1 Study group and design

From March 2005 to January 2007, 155 TB-exposed health care workers were included from three major University Hospitals in Norway: Haukeland University Hospital (HUS), Ullevål University Hospital (UUS), and Akershus University Hospital (AHUS). For inclusion in the study, exposed persons had to be in close contact (stay in the same room) with a sputum-smear positive pulmonary TB patient in a non-protected manner for at least 1 hour. Subjects were then grouped according to the time of exposure: 'Low exposure' was defined as 1 to 8 cumulative hours of close contact, while more than 8 cumulative hours of close contact was considered to be 'high exposure'.

A control group of 48 non-exposed individuals were recruited from the non-clinical staff at AHUS. Only individuals without any known prior exposure were

included in this group. The mean age of the control group was 41 years, with a female:male ratio of 33:15. The exposed group had a mean age of 39 years, with a female:male ratio of 133:22. Ten employees came from TB high-endemic countries. Both groups lived in the same geographical area and consisted of employees at Norwegian hospitals with middle incomes. The exposed group was followed up according to the national guidelines, including TST and chest X- ray independent of the T-SPOT.*TB* test result [159]. All participants answered a questionnaire concerning BCG vaccination status, former TB, previous exposure, and residency in TB high-endemic countries. Previous studies have demonstrated that individuals infected with TB complete their cellular immune response within 8 weeks after exposure [217]. Thus, both the T-SPOT.*TB* test and the TST were performed as close to 8 weeks after exposure as possible (mean, 11.5 weeks). The subjects had not been tested previously by an IFN-γ test, so their pre-exposure T-SPOT.*TB* status was not known. The last documented TST found in the hospital records and TST results obtained 8 weeks after exposure were used as the basis for determining infection status. The study was approved by the Regional Committee for Medical Research Ethics East (REK Øst). Informed consent was obtained from all participants, clarifying that follow up and treatment would be offered regardless of participation and according to national guidelines [159].

Tuberculin skin test

TSTs of both exposed personnel and controls were performed according to the Mantoux method with Purified Protein Derivate (PPD) RT 23 SSI, (2 TU) from Statens Serumsinstitut (SSI), Copenhagen, Denmark. Transverse induration in mm at the injection site was measured after 48-72 hours, and the results were interpreted according to the national guidelines [159]. Reading of test results was repeated if the induration was large, showed signs of adverse reactions, or was difficult to read.

T-SPOT.*TB* test

The T-SPOT.*TB* test, (Oxford Immunotec, UK), was used according to the manufacturer's instructions. The blood was always drawn prior to the TST to avoid any possible interactions caused by rapid homing of specific T-lymphocytes to the

tuberculin injection site. Venous blood drawn into Cell Preparation Tube (CPT) vacutainers (Beckton Dickinson, Oxford UK) was sent by courier service to the Norwegian Institute of Public Health in Oslo, and analysed within 6 hours. In brief, peripheral blood mononuclear cells (PBMC) were isolated from blood following centrifugation, washed and counted. PBMCs at a concentration of 250,000 cells/well in AIM V® cell culture medium (Invitrogen Corporation, Carlsbad, USA) were stimulated with ESAT-6 and CFP10 in 96-well plates pre-coated with anti-IFN-γ capture antibodies, and incubated overnight at 37^{o}C in 5% CO_2. Medium only and mitogen (Phytohemagglutinin) were used as negative and positive controls, respectively. The next day the T-SPOT.*TB* assay was developed by adding an alkaline phosphatase-conjugated detection antibody and substrate. Coloured spots, representing individual INF-γ-producing T cells, were counted manually using a microscope. The results were recorded based on the definition of positive and negative reactions given in the instructions from the manufacturer. All initial positive results were confirmed by analysis of a second blood sample before they were reported as positive.

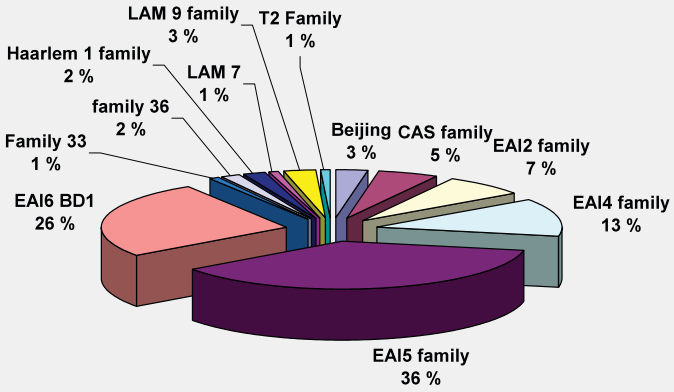
Data handling and statistical analysis

All data were entered into a central Microsoft Access™ database, approved by the Norwegian Data Inspectorate, and statistical analysis was performed with the Graph Pad Prism 4™ software.

3. RESULTS

Paper I

A total of 780 patients were positive for *M. tuberculosis* by sputum-smear microscopy at the BRAC TB clinics in Sunamganj district (Chatak, Derai, Duarabazar and Sunamganj Sadhar upazillas) during the study period. Out of these, the research team managed to collect 267 sputum specimens which were sent to ICDDR'B in Dhaka. *M. tuberculosis* was isolated from 111 (42%) of these samples. The positive cultures were isolated evenly throughout the year. Of the 111 isolates, 5 carried less than 5 copies of IS*6110*. A high degree of diversity was found in the collected bacterial population. A total of 105 genotypes were observed among the isolates. Also, contrary to that commonly observed in studies from urban areas of South East Asia, only three strains carried a IS*6110* RFLP pattern and spoligotype pattern that was typical to those of the BL. The large genetic diversity found by IS*6110* RFLP analyses suggested that the majority of TB cases in this isolated, rural area, of a high incidence country, represented an established epidemic and was dominated by three main groups of *M. tuberculosis*. Each of the three main groups of *M. tuberculosis* isolates that were demonstrated in the current study represented a genetic diversity of more than 60%. This suggested that the isolates within these groups had evolved in parallel



The distribution of *M. tuberculosis* families in 111 TB patients of the Sunamaganj District of Bangladesh analysed according to the SpolDB4 database.

and that each group represented an established epidemic.

These findings were confirmed by the spoligotyping. At the time of publishing of article 1, the SpolDB4 was not available. Analyzing our data with the SpolDB4 38 (34%) of the isolates were clustered into 8 shared types, whereas 74 strains (66%) were unique. The strains were clustered into 8 major clades: 8% percent (3 BL and 6 CAS) of all strains belonged to the modern principal genetic group 1 (PGG 1), 5.0% (2 Haarlem 1, 1 LAM 7 and 3 LAM 9) belonged to PGG 2 and 3% belonged to the "miscellaneous" PGG 3. However, the major dominating clade was the "ancestral" East African-Indian (EAI) clade. The low diversiy among non-identical isolates suggested that this represented an established epidemic. Also, it appeared that this epidemic only to a minor degree was influenced by recently imported and recently disseminated strains. Of the total strain population 8 (7%) belonged to the EAI 2 clade, 14 (13%) to the EAI 4 clade, 41 (36%) to the EAI 5 clade and 29 (26%) belonged to the EAI6 BD1 clade.

Paper II

From November 2003 to December 2004 a total of 268 ss+ samples were collected. *M. tuberculosis* could be recovered from 148 of these samples. Both fingerprinting and even more so the DST suffered from substantial contamination of the strains on arrival from Bangladesh. Despite repeated decontamination procedures utilizing sodium laurylsulphate [218], DST results were produced for only 95 out of the 148 isolates. The MGIT 960 system was utilized. Total resistance among new cases to any drug was 31%. Among new cases resistance to SM was found in 17% of the isolates, 23% were resistant to INH, 2% to RMP, 12% to EMB and 2% (2 cases) were MDR-TB. There were 11 previously treated cases. Among these, 3 were resistant to SM and 4 to INH. Total resistance to any drug, among all cases (new and previously treated cases) was 32%. A total of 18% of all cases were resistant to SM, 24% to INH, 2% to RMP, 11% to EMB, and 2% of all cases were MDR-TB.

The highest rate of resistance was found for INH (24%), followed by SM (18%). Resistance to two or more drugs was seen in 10.5% of the cases, but the most unexpected finding was the relatively high resistance to EMB (12%).

Paper III

Early diagnosis and immediate initiation of treatment are essential for an effective TB control program. Delay in diagnosis is significant to both disease prognosis at the individual level and transmission within the community. Most transmissions occur between the onset of cough and initiation of treatment. Paper III describes a systematic review of 58 studies addressing delay in diagnosis and treatment of TB. We found different definitions of, for example, debut of symptoms, first appropriate health care provider, time to diagnosis, and start of treatment. Rather than excluding studies that failed to meet strict scientific criteria, we tried to extract the "solid findings" from all of them to arrive on a more global understanding of diagnostic delay in TB.

The main factors associated with diagnostic delay included human immunodeficiency virus; coexistence of chronic cough and/or other lung diseases; negative sputum smear; extrapulmonary TB; rural residence; low access (geographical or sociopsychological barriers); initial visitation of a government low-level healthcare facility, private practitioner or traditional healer; old age; poverty; female sex; alcoholism and substance abuse; history of immigration; low educational level; low awareness of TB; incomprehensive beliefs; self-treatment; and stigma.

The core problem in delay of diagnosis and treatment seemed to be a vicious cycle of repeated visits at the same healthcare level, resulting in nonspecific antibiotic treatment and failure to access specialized TB services. Once generation of a specific diagnosis was in reach, TB treatment was initiated within a reasonable period of time.

Paper IV

The median infectious period for retreatment cases, defined as the time from cease of primary treatment to start of new treatment, was 60 days. It was estimated that approximately 33% of all new ss+ PTB cases were undiagnosed or not notified by the NTP. The estimated infectious period of each undiagnosed case was 60 days, thus these cases contributed 150,360 days to the infectious pool. There were 75 retreatment cases contributing 3,675 days to the infectious pool. There were also 60 treatment failures, which because of their long infectious period of 365 days, made a substantial contribution of 21,900 days to the infectious pool. There were 5,013 new ss+ PTB cases, each with a median TD of 95 days. These cases made the largest contribution to the infectious pool (476,235 days). The largest group of patients was the

6,159 ss- PTB cases who initiated TB treatment. Estimating their infectiousness as 20% of that of the ss+ cases, they had an infectious period of 20 days and contributed 123,180 days to the infectious pool. The total estimated infectious pool for the Amhara region in 2003 was 775,350 days, or 4,284/100 000 pop.

Paper V

Among the 155 exposed health care workers tested, 27 individuals were defined as new TST positive cases after recent exposure, while only 3 of these had a positive T-SPOT.*TB* test. There were no indeterminate test results, and no excluded HCW or T-SPOT results. The number of T-SPOT.*TB* positives represents 11% of the individuals defined as new TST positives after exposure (3/27) and 2% of the total number of exposed people tested (3/155**)**. In addition, 15 individuals had been previously defined as TST positive before exposure. Only 2 were T-SPOT.*TB* positive: one was born in a TB high-endemic country, and the other had been previously treated for pulmonary TB. Interestingly, the former had a negative TST at the time of immigration to Norway, raising a question concerning the source and place of infection.

All individuals detected as T-SPOT.*TB* positive belonged to the infected group, and percentage concordance between T-SPOT.*TB* and TST, including both previous and new TST positives, was 12% (5/42). Thus, the frequency of latent TB in the total cohort was 3% (5/155), whereas the TB transmission rate in the actual TB exposure study was estimated at 2 % (3/155). The 48 control participants were all T-SPOT.*TB* negative, but 3 persons in the control group were TST positive.

The average time between first exposure and testing was 11.5 weeks. Among the exposed individuals, 51 participants belonged to the “high exposure” group (> 8 hours), and 104 participants fell into the “low exposure” group (≤ 8 hours). There was no correlation between length of exposure and TST results. In addition, there was no correlation between T-SPOT.*TB* positivity and TST results. For the 3 T-SPOT.*TB* positive individuals infected after known exposure, two were exposed ≤ 8 hours and one was exposed > 8 hours.

4. DISCUSSION AND CONCLUSIONS

4.1 MOLECULAR EPIDEMIOLOGY OF M. TUBERCULOSIS IN BANGLADESH

4.1.1 A high degree of diversity and predominance of the "ancestral" East African-Indian clade

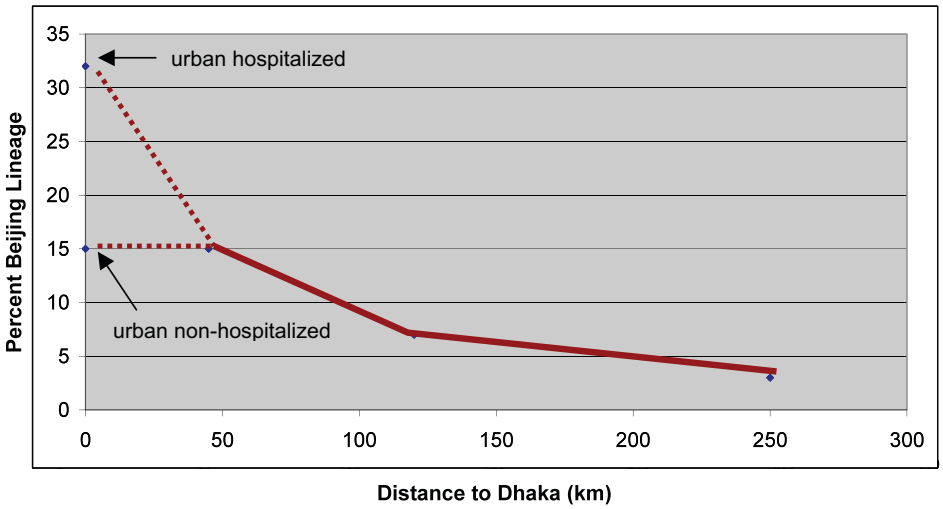
In the current study, a high degree of diversity among the *M. tuberculosis* population and low prevalence of the Beijing genotype was demonstrated in a rural area of Bangladesh (Paper I). The local *M. tuberculosis* population was dominated by the the EAI clade. These findings have also been confirmed by other studies in Bangladesh where 44-56% of *M. tuberculosis* collections have been assigned to the EAI linage [4, 117, 219]. Most strains of the EAI clade could be classified into EAI1 to EAI5. Within the EAI clade, 49 strains of the current collection had the spoligotype signature designated EAI6-Bangladesh 1 (EAI6-BGD1). It thus appears that "ancestral" TB clades like the EAI [40] are predominant in rural Bangladesh. This indicates that these strains have been present in the area for long periods and may thus have been endemic for many decades. The large diversity found by IS*6110* RFLP analyses also suggested that the majority of TB cases included in our study represented an established epidemic since it was dominated by three diverse groups of *M. tuberculosis* where most isolates had at least one other closely related strain. The spread of TB in the Sunamganj district was not caused by any predominant individual strain, thus a significant contribution to the TB burden in this highly endemic country seems to be caused by endogenous reactivation. Although a large genetic diversity has also been demonstrated in a rural area of India [220], these findings are opposed to the findings of many urban-based South East Asian studies that have found that Beijing genotypes contribute to major outbreaks and transmission hot-spots of TB [134, 221].

4.1.2 Our study could indicate that the recent global dissemination of the Beijing genotype has not yet reached remote areas of Bangladesh

From 2001 to 2003 Rahim et al. collected 224 *M. tuberculosis* strains from non-hospitalized patients from Dhaka and Matlab, a rural area 45 km southeast of Dhaka. The BL comprised 15% of the strains, both among the rural and urban patients

[117], compared to 32% BL found by Banu et al. among TB patients in a Dhaka hospital [4]. The higher BL proportion among the hospitalized TB patients could be due to the selection of resistant cases, but is else difficult to explain. The equal distribution of BL between Matlab and Dhaka in the study by Rahim et al. could indicate that a distance of 45km does not make a substantial difference. The split dotted beginning of the figure below depicts the difference of BL prevalence in Dhaka found by Banu et al. (32%) and Rahim et al. (15%).

Beijing lineage as a function of distance to Dhaka



A study from Mymensingh, 120km North of Dhaka, found 7% BL strains [219], and our study from Sunamganj, which is 250km North of Dhaka, found 3% BL. It seems that the further away from Dhaka, the less spread of BL strains. Our findings indicated that the recent global dissemination of the Beijing genotype has not yet reached remote areas, such as Sunamganj of Bangladesh. If the Beijing genotype is heterogeneously spread throughout areas in high-incidence countries, it lends support to the hypothesis that this family represents a relatively new epidemic and that the Beijing genotype may fuel a current change in the global *M. tuberculosis* population.

Other possible contributing factors to the low presence of BL in isolated rural areas could be the absence of drug pressure (until recently), and the lag in shift towards new strains caused by latency or that the BL requires higher CCS than other lineages.

For the rural areas of Bangladesh a consequence of our knowledge of the epidemic situation must be to give first priority to prevent new emerging strains from

spreading. The previously described decentralized and community based BRAC model (1.9) with high case detection and treatment completion rates is an outstanding example of how this can be achieved.

4.2 DRUG RESISTANCE IN THE SUNAMGANJ DISTRICT

4.2.1 Presence of MDR-TB as expected - standard treatment regimen for TB still effective for the majority of the ss+ cases.

The rate of MDR-TB (2%) was higher than that identified in rural areas of Mymensingh, both in 1995 and 2001 [222], but due to the limited number of isolates in our material the difference is not significant (Paper II). A low prevalence of RMP resistance (1.1%) and MDR-TB (0.4%) for new cases was demonstrated in 1995 before DOTS was established in Bangladesh, and these levels dropped after the introduction of DOTS [182, 223]. The same success with DOTS has been observed in BRAC operated rural areas like Sunamganj [224]. In Matlab, another rural area, also studied after the introduction of DOTS, MDR-TB was absent among new cases, while it was common (27.3%) among previously treated cases [184]. Studies from rural India (1997-2003) show that primary resistance to RMP is 0.5-4.4% and that the prevalence of MDR-TB is 0.5-3% [185]. The same range of frequencies of MDR-TB among new cases are described in Myanmar (4.2%) [186], Thailand (4.2%) [187], and China (4.5%) [188]. The same levels of MDR-TB have also been described in the urban areas of Dhaka [184, 225, 226]. The frequency of MDR-TB found in Sunamganj was therefore not unexpected, since it corroborated to previous studies. Together, these studies indicate that the prevalence of MDR-TB is higher in urban areas of South East Asia than in rural areas. Also, MDR-TB is more common among retreatment cases than among new cases. Our study indicated that the NTP of Bangladesh has adopted a standard treatment regimen for TB that is still effective for the majority of the ss+ cases.

4.2.2 High rates of resistance to isoniazide, streptomycin and ethambutol

The high rates of resistance to INH (24%) and SM (18%) presented in Paper II, most likely reflected that a majority of the current patients were infected many years

ago, when the most prevalent drugs SM and INH were frequently used in monotherapy [180]. This should imply a low rate of recent transmission which is in accordance with what was demonstrated by use of IS*6110* restriction fragment length polymorphism (Paper I). Among the isolates within this population of *M. tuberculosis* most originated from patients found not to have been recently infected [209]. Since the most potent bactericidal effect of INH is in the early phase of treatment [227], resistance to this drug is an important indicator of the emergence of drug resistant TB. An increased risk of treatment failure has also been demonstrated in areas with high levels of INH resistance. A high proportion of INH resistant strains may develop resistance also to RMP [228, 229] and thus acquire MDR-TB. The high level of INH resistance identified in the current study was therefore alarming and justifies follow up studies as well as a close monitoring of the DST levels among *M. tuberculosis* isolates of the Sunamganj area of Bangladesh.

The relatively high rate of resistance to EMB (12%) found in the current study was similar to the substantial rate of resistance to EMB found in Matlab (14 %) [184]. In Mymensingh however, resistance to EMB was not found [182, 223]. We believe these discrepant results indicate that marked regional differences exist in EMB susceptibility among the various local *M. tuberculosis* populations in Bangladesh. It may however, also reflect the non-standardized techniques related to performing and interpreting sensitivity to EMB.

In conclusion, our study demonstrated that DST is needed and possible in low infrastructure areas of developing countries like Sunamganj in Bangladesh. A prompt response to the world-wide increasing drug resistance must be to establish DST facilities. From an epidemiological point of view, to treat MDR-TB cases with first line drugs will rapidly deteriorate the situation. A major task must be to establish well-organised transportation systems and quality-assured chains of DST facilities.

4.3 LOGISTICS AND VALIDITY OF THE SUNAMGANJ STUDY

The current studies raised some questions related to diagnosis, molecular fingerprinting and drug susceptibility testing of *M. tuberculosis* isolates from remote areas like Sunamganj. The field staff participating in the current study could only be available for sputum collection for 268 of the 743 ss+ TB cases diagnosed during the study period in these upazillas. The sputum samples that were collected could not be processed on the day of sampling. The current area is only accessible by small boats

during most parts of the year. Samples were kept in refrigerators, but between points and overnight during transport to ICDDR,B had to be stored in cooling boxes. Traore et al. found that the loss of mycobacteria was minimal in sputa stored at 2-4^0 C for up to 14 days. At 37^0 C however, they observed a complete destruction of mycobacteria present in the samples [230]. Despite attempts to maintain refrigeration all the way to Dhaka it cannot be ruled out that some strains were lost during this transport. It appears likely that the loss of *M. tuberculosis* cultures due to unavailability of field staff, local storage prior to shipment, and exposure to non-refrigerated temperatures was random. In such cases, it is unlikely that specific groups of *M. tuberculosis* with particular fingerprints or sensitivity patterns are favoured. We therefore believe that the isolates were representative of the TB epidemic of Sunamganj.

4.4 REASONS FOR DIAGNOSTIC DELAY IN THE DIAGNOSIS AND TREATMENT OF TB

4.4.1 Major findings

Our analysis demonstrated that the interacting factors affecting patients' health-seeking behavior and the availability of TB diagnosis and treatment can be categorized as involving either the patient or health care system. Most factors influenced both patient and system delay, but some factors were more closely related to patient delay: alcohol or substance abuse, poverty, low access to health care facilities, rural residence, old age, belonging to an indigenous group and incomprehensive attitudes, beliefs and knowledge about TB. Other factors were evidently more linked to health care delay: coexistence of chronic cough and/or other lung diseases, having extrapulmonary or negative sputum smear TB, less severe and indifferent symptoms or absence of haemoptysis, poor health care infrastructure and seeking traditional and private practitioners first. As patients continue to go untreated, absent isolation, both components equally contribute to the infectious pool.

Important factors related to patient delay
alcohol or substance abuse poverty low access to health care facilities rural residence old age belonging to an indigenous group attitudes, beliefs and knowledge
Important factors related to health care delay
chronic cough other lung diseases negative sputum smear TB extrapulmonary TB less severe and indifferent symptoms absence of haemoptysis poor health care infrastructure seeking traditional and private practitioners first
The vicious circle of repeated visits at the same level is the core problem of diagnostic delay

4.4.2 Seeking a traditional practitioner first causes delay

A range of studies have shown that selection of a traditional practitioner for the first visit is associated with a prolonged delay in diagnosis [231-240]. In Yimer et al.'s study from Ethiopia, patients who first visited a qualified medical provider experienced a 21-day delay before initiation of treatment. Patients who first visited a traditional healthcare provider waited shorter (15 days) before first seeking healthcare, but the period from first visit to initiation of TB treatment was 4-fold greater than for the patients who visited a qualified medical provider first [199]. These findings are sup-

ported by studies from The Gambia [238], Tanzania [231], and Penang (Malaysia) [241].

4.4.3 Poor access to the local NTP and stigma

Many studies describe a bottleneck in reaching the local unit of the NTP. Multiple studies observed poor access to the NTP as one of the main factors in delayed diagnosis [15, 199, 231, 232, 235, 237, 239, 242-250]. In many high endemic countries, it appears crucial that a unit of the NTP is within a 1-day walking distance, as many patients have limited access to motorized vehicles. In addition to geographic distance, the studies identified several other barriers. One of the most important barrier is stigma [232]. Many patients were highly reluctant to visit the NTP facility, because it would mean disclosing to the public that they had TB. Even worse, in many countries, TB is so closely linked to HIV that patients fear they are revealing their HIV status to their neighbours [240]. Also, many of the NTP personnel are unfriendly. Patients feel deprived of both privacy and dignity. The widespread introduction of DOTS also means that patients must visit daily so that their consumption of medication can be directly observed; this is perceived as humiliating, time consuming, and a threat of a substantial loss of income [199, 251, 252]. A marginal farmer or a day labourer will often have to choose between treatment and placing food on the table for his family. Most of the studies from developing countries demonstrated a significant link between delayed diagnosis and poverty.

4.4.4 The non-specific nature of symptoms and self-treatment

Most of the studies in our review also demonstrate the non-specific nature of symptoms and the disease's first natural history as a core problem [201, 243, 245, 248, 253, 254]. In The Gambia, TB is often misdiagnosed as malaria or viral infections [238].

Several studies also highlight the problem of incorrect self-diagnosis including incorrect self-treatment [199, 233, 246]. In the study in Pakistan, more than 50% of patients practiced self-treatment, and 42% first searched a pharmacy for their symptoms [232]. Many studies link this to the problem of stigma, in fact the unhappy triad of incomprehensive beliefs, low awareness, and stigma.

4.4.5 The vicious circle of repeated visits at the same level is the core problem

Our analysis revealed that the vicious circle of repeated visits at the same level is the core problem of diagnostic delay in most high endemic countries. TB is a rare disease, and more than 95% of patients with chronic cough seeking treatment at the level of primary healthcare do not have TB [153]. The delay in diagnosis on the part of health providers does not necessarily reflect inferior performance, but instead a lack of effective diagnostic tools and follow-up routines. Correct diagnosis requires both good training and available diagnostic facilities. The number of repetitions of this vicious cycle proved to be highly dependent on both the patient's beliefs and awareness of TB and the category of healthcare provider. In most countries, private practitioners are consulted first, and this pattern is significantly associated with a longer diagnostic delay. This pattern is also recognized as a source of bottleneck in low endemic countries. A New Zealand study observed that the first consulting doctor often did not perform a chest X-ray, asked about previous TB, or obtained a sputum smear, although the patients had classical symptoms [255]. The same observations were made by Ward et al. in Australia [256]. Cough was treated symptomatically, and CXR were misdiagnosed.

4.4.6 Why reduction in diagnostic delay should be a core target of all TB control programs

Emphasis on the reduction of DD is not just an academic exercise; it is a crucial task of all TB control programs. The nature of epidemics is that their reproductive rate has a dramatic impact on the epidemiological trend. Until the twentieth century, the reproductive rate for the TB epidemic was above 1.0 in Europe and North America [257, 258] . Small changes in the host-bacterium balance such as improved housing caused the TB epidemic to decline, long before specific treatment and preventive measures were available. In these same parts of the world, symptomatic sexually transmitted infections almost disappeared when effective antibiotics became available, with a sharp decline in the reproductive rate as the TD was reduced [259]. The same changes should occur with regard to TB, and the crucial task is to reduce the unnecessary long treatment delay. A majority of the TB patients have been in touch with

the formal health services as early as two weeks after the start of the symptoms, but they are not identified. More than 95% of the patients who seek health care for chronic cough do not have TB [255]. How do we identify the 5% who have TB at the first consultation, thereby preventing each from spreading TB? A crucial challenge is the lack of effective screening tests that are inexpensive and feasible at the primary health care level. If TD can be reduced and well organised specialized TB services are made available for all patients, the current rising world-wide epidemic may be reversed.

4.5 THE UTILIZATION OF TREATMENT DELAY TO MONITOR THE INFECTIOUS POOL OF TB IN A POPULATION

As described previously, we theorized that a systematic recording of TD can be utilized as a feasible tool to monitor the size of the infectious pool at the local level. In our Ethiopian study we aimed at verifing this tool in a high endemic area. However, there are several demanding variables in our calculations, and substantial methodological challenges that need to be addressed.

4.5.1 Size and nature of the undiagnosed group

The WHO calculated the CDR for new smear positive cases in Ethiopia to be 36% in 2003. As discussed in the background chapter, the CDR is built on old tuberculin surveys extrapolated to the present situation [104]. For several reasons it is unlikely that there are 64% undiagnosed cases (WHO). First, pulmonary TB is generally a serious disease with characteristic symptoms. The patient seeks treatment, self-cures, or dies within a few months [101]. In many communities, the traditional belief as well as the experience of previous generations is that if you get TB you will die [260]. After the introduction of modern medicines these beliefs seem to be changing. Despite poverty and long distances to healthcare, most patients seek treatment, and they prefer allopathic medicine [261]. Our study on help-seeking behaviour in rural Bangladesh found that more than 90% of patients with symptoms of TB preferred to visit an allopathic doctor first. Among the ten surveyed diseases, this was the one with the highest preference score for allopathic medicine [261].

To estimate the size of the undiagnosed group a key question is: Are the non-detected mostly not-ever detected, or are they not-yet detected? After DOTS is introduced in a country, the number of new cases identified proceeds through distinct tran-

sitional phases. This is illustrated by the development in The Sudan [260]. The national strategy was to first establish TB treatment facilities in the larger cities and main hospitals. This was followed by a steep rise in the number of diagnosed cases. After the introduction of the NTP at the district level, a further steady increase was expected, but this did not happen. Instead, after an initial small increase in the case finding, mainly involving women and children, a plateau was reached. The symptoms of TB were probably serious enough that patients travelled a long distance for care. Thus the main proportion of patients were diagnosed at a time when treatment facilities were only available in the main cities [260]. This indicates that most of the un-notified TB patients are not-yet detected rather than not-ever detected. They come forward and are notified sooner or later.

Ethiopia is an important model to observe, because TB drugs are not available outside the NTP. Private practitioners as well as traditional practitioners all refer their TB patients to the NTP for treatment [199]. This makes Ethiopian data on the undiagnosed group more reliable than data from most other high endemic countries were TB drugs are sold on the open marked and a range of practitioners outside the NTP are providing treatment. A prevalence study was conducted by Shargie et al. in an area of Ethiopia with a well organized TB control program that had functioned in more than ten years [262]. To the authors' surprise they found that only one third of the total number of new ss++ where undiagnosed by the local TB control unit.

This is further illustrated by an Ethiopian intervention study wherein 32 local communities were randomly allocated to a control group of passive case finding or an intervention group of active case finding through monthly diagnostic outreach clinics, house visits, and public gatherings. In the intervention area, case finding was not significantly increased, but the TD was shortened. The proportion of patients with more than 3 months of symptoms was 41% in the intervention group and 63% in the control group. A sputum survey of 16,697 adults from the same area revealed a 2:1 ratio of ss+ cases receiving treatment to newly detected ss+ cases. The prevalence of ss+ TB was surprisingly low, only 78 per 100,000 [198, 262]. These studies were performed 10 years after the introduction of an effective DOTS program with 75% coverage and a 73% treatment success ratio [263].

Given that the non-detected patients are mostly not-yet detected in the process of reaching a diagnosis, 33% additional non-detected patients is in accordance

with the above mentioned studies, and we utilized this number as the base of our calculations of the size of the undiagnosed group.

In general, the relative number of un-notified cases, and the period they remain untreated differs between countries and the regions within them, and must be carefully estimated before calculating the infectious pool.

4.5.2 Contribution of the sputum smear-negative cases

Sputum- smear negative (ss-) PTB cases are generally less infectious, and their contribution to the infectious pool is not well studied. We based our calculations on two molecular studies: First a study in Greater Vancouver, Canada, which determined that ss- cases are responsible for at least one sixth of culture-positive episodes of TB transmission [264]. Secondly a study in San Francisco, California found that the source patient was ss- in at least 17% of the registered episodes of transmission, and it was estimated that ss- patients were 22% as likely as ss+ patients to transmit TB [46]. Studies like these, which demand an almost complete overview of the transmission in a population and the utilization of advanced molecular techniques, have not been performed in any high endemic country. However, we have no reason to believe that the infectiousness of ss- cases should be different from low to high endemic areas. Thus, in our estimate of the infectious pool ss- PTB cases contribute to the infectious pool as 20% of the ss+ PTB cases, and we have divided the ss- PTB cases' TD by 5 to estimate their contribution to the infectious pool.

4.5.3 Infectious period after start of treatment

Two recent studies estimated the median time to sputum culture conversion after first-line treatment to 28 days [265] and 33 days [266], but the viability of the organisms rapidly deteriorate after treatment onset [267]. From several studies conducted in the 1960s a practical conclusion was agreed that ss+ PTB patients remain infectious till on average two weeks after start of treatment [268, 269]. Taking into account the progressively reduced infectiousness, our estimate has added an infectious period of 15 days to each ss+ patient after the start of treatment.

4.5.4 Defining the onset and end of the infectious period

Patient's delay (PD) was defined as the interval between the onset of any symptom and the time at which the patient sought contact with any healthcare provider outside the household, including traditional practitioners. We chose to define the end of the delay as the day the patient started on treatment. Treatment delay (TD), which defines each patient's contribution to the infectious pool, was thus defined as the interval between debut of any symptom and the start of treatment. We justify this approach based on the fact that the infectious period is the period during which the patient coughs, and according to multiple studies, coughing is the cardinal first symptom of approximately 95% of ss+ patients [199, 270]. Usually, the patient can accurately define the onset of their symptoms, and even distinguish a new coughing pattern from previous coughing patterns with other causes such as COPD or smoking [245]. The first symptom is important information and can be obtained with little extra effort by adding a question to patient registration forms. Moreover, this variable is not easily susceptible to "target perversion", which means that it is not so easy to manipulate to fulfill official demands and goals; it is information provided directly by the patients and recorded in the NTP files.

4.5.5 Conclusions

We found that by addressing systematically the challenges of each variable in our calculations we obtained a realistic estimate of the infectious pool of TB in the population. Although there are still questions to be answered and more research to be done regarding the accuracy of the calculations, there is one application that is particularly usefull, especially at the local level; to monitor the trends. Still the validity of our approach needs to be confirmed. But, provided the recording is done systematically over time, *changes* in the infectious pool should be possible to obtain with high levels of accuracy, and be of substantial value, especially in monitoring epidemiological trends and the performance of the local TB program.

4.6 USE OF INTERFERON GAMMA-BASED ASSAY TO DIAGNOSE TUBERCULOSIS INFECTION IN HEALTH CARE WORKERS AFTER SHORT EXPOSURE

A positive TST has previously been considered the gold standard in screening for *M. tuberculosis* infection. However, there is currently no definitive way to decide whether a person is latently infected with *M. tuberculosis*. A recent prevalence study by Soborg et al. in a Danish hospital confirmed earlier findings that TST is hampered by low specificity in BCG-vaccinated individuals [271, 272]. The authors found a 34% prevalence among TST-positive HCWs, but the only significant risk factor associated with a positive TST was prior BCG immunisation; no association was observed with other important risk factors, such as occupational exposure to TB or hospital staff position [271]. Other studies in low-endemic countries have also found that a positive TST test is primarily associated with prior BCG vaccination [273]. Nevertheless, how can we conclude that the low post-exposure rate of infection detected by T-SPOT.*TB* in our study represents the real situation if there is no gold standard? One possible strategy is to estimate the likelihood of having latent TB infection by calculating a contact score that quantifies exposure to and infectiousness of the index case, as was done by Shams et al. [274]. A range of other studies also provide extensive evidence that the IGRAs correlate better to exposure than does TST. Therefore, we based our conclusions regarding the prevalence of TB infections on the T-SPOT.*TB* test [167, 273-279].

Of 155 exposed HCWs and 48 healthy controls, all but one had a visible scar from BCG immunisation, which has been compulsory in Norway at the age of 14 until recently. Norwegian legislation also demands that all HCWs are asked for a certificate of TST at the time of appointment, and if the existence of a recent TST cannot be documented then a new TST is performed. The high correlation between BCG vaccination and a positive TST and the high specificity of the T-SPOT.*TB* test for *M. tuberculosis* infection (98%) make it likely that these are mostly false positive TST reactions rather than false negative T-SPOT.*TB* reactions. In addition, the boosting effect of repetitive skin testing in health personnel may also contribute to the somewhat surprisingly low concordance between infection status according to T-SPOT.*TB* and TST that was observed in this study (12%). The accuracy of the T-SPOT.*TB* test is also supported by the fact that all five T-SPOT.*TB*-positive individuals also had a strong positive TST. Previous studies indicate that T-cell–based assays such as T-SPOT.*TB* and QFT are

more specific than TST, as they are not confounded by previous BCG vaccination or exposure to a large majority of other mycobacteria [167, 273, 279, 280]. Still, there is no diagnostic gold standard for latent TB and the fact that 89% of employees recently defined as infected by TST tested negative with the T-SPOT.*TB* test calls for further studies of kinetics and immune mechanisms in TB infection.

In contrast to most other studies related to TB transmission within health institutions, we have in this work compared the performance of T-SPOT.*TB* and TST in a group of HCWs with well-defined short-term exposure to contagious TB patients in a hospital setting in a TB low-endemic area. In addition, the results have been used to evaluate the role of IFN-γ release assays in improving the surveillance of TB transmission to health personnel in a low-incidence country like Norway.

Provided that the T-SPOT.*TB* results are the most reliable compared to TST results, our study indicates that the risk of infection among health care workers after short-term exposure to TB patients in a hospital setting is low (2%). This somewhat contradicts the findings of a Swiss long-term institutional study in which 15% of contacts were T-SPOT.*TB*-positive after prolonged unprotected exposure [273]. Although both studies were performed in health care institutions in low incidence countries, the exposure time may account for the observed differences in transmission. A study from Denmark also reported a low proportion (1%) of latent TB among HCWs as detected by the QFT test [271]. However, these data were not based on recent exposure, but rather represent the general prevalence level among hospital personnel working in departments with TB patients. We found a prevalence level of 3% in our cohort. Not surprisingly, these results are in contrast to findings from a high-endemic country like Russia, where a study utilizing QFT revealed a prevalence level of 41% among hospital staff working with infectious diseases [136]. Several reports based on TST conversion indicate that the risk of being infected may be high, even within a limited time frame of exposure [10, 281]. This has also been confirmed by a T-SPOT.*TB* study in Italy in which 32% of the staff in a maternity ward became positive for TB after a mean exposure time of 6 hours [280]. Compared to these findings, our study detected a low degree of transmission. However, it should be noted that the majority of the individuals in this study had been exposed for less than 8 hours before precautions were taken. Still, among the three persons with positive T-SPOT.*TB* tests (believed to be recently infected), two were exposed for less than 8 hours. The absence of a statistically significant correlation between exposure time and both TST and T-SPOT.*TB* results is

probably due to both the low exposure time and the small number of participants, since these correlations have been demonstrated in many other studies [10, 280, 281].

Importantly, the results indicate that using IFN-γ release assays as an alternative to the present follow-up strategy based on TST results could save substantial resources. Although TST by itself will be less expensive than a comprehensive laboratory procedure, the utilization of *M. tuberculosis*-specific blood tests has the potential to save major resources as the number of persons who must be followed up for 3 years can be reduced by up to 89%. In this context, the possibility of avoiding unnecessary and costly treatment, including serious side effects, is also of considerable importance. In addition, most exposed health care workers will avoid long-term anxiety by obtaining a negative result at an early investigational stage. Finally, the small number of infected persons who require treatment can be identified immediately.

Oxlade et al. performed a 20-year cost-benefit analysis that used Markov modelling to compare the costs of TB screening with different strategies among hypothetical cohorts of foreign-born immigrants to Canada and contacts of TB cases. Model inputs were derived from published literature and utilization of the QFT test. For entering immigrants, screening with Chest X-Ray would be the most cost-effective and QFT the least cost-effective strategy. Sequential screening with TST followed by QFT was more cost-effective than either QFT or TST alone. In contact tracing after exposure, the most striking finding was that screening with TST followed by QFT, if positive, was more cost-effective than any other strategy. This was largely because TST alone was not effective if the exposed group had been vaccinated with BCG after infancy [282]. These findings were also confirmed in a Swiss study by Wrighton-Smith et al., estimating the costs of screening a cohort of 1000 individuals for latent tuberculosis; screening with TST alone followed by Chest X-Ray and clinical follow ups of the positive cases was estimated to €695820; T-spot.*TB* alone was estimated to €387135; TST followed by T-spot.*TB* of the positives was estimated to €342563, i.e. the less costly [283].

Due to the fact that BCG immunization has been administered routinely in Norway, specific blood tests should be introduced in all post-exposure contact tracing situations. Because TB transmission to health personnel in Norway seems to be rather low, the two-step screening approach (TST followed by IFN-γ release assay) might be attractive, and should be considered more closely from a cost-benefit perspective. It should also be noted that the QFT method, although less sensitive in immuno-

suppressed individuals, has both logistic and economic advantages compared to the T-SPOT.*TB* assay, and implementation of the QFT has recently been suggested in Norway's national guidelines. The introduction of specific T-cell based assays for post-exposure screening and subsequent prophylactic treatment will become a rational and important component of the national TB control strategy.

5. CONCLUSIONS

We showed that a well established epidemic in rural Bangladesh represented a low rate of recent transmission. A majority of the strains belonged to the ancient East African- Indian (EAI) lineage (Paper I).

Our study found that the total resistance among new cases to any drug was 31%, 2% were MDR. The NTP in Sunamganj is still effective, although the high resistance to INH is alarming (Paper II), and DST services were possible and highly needed.

Due to the low rate of transmission in the high-incidence area, it was likely that most patients had been infected a long time ago. We thus wanted to measure the impact DD could have in similar high insidence settings (Paper IV). In order to study the impact of DD, an introductory review of published studies describing DD in numerous countries was conducted (Paper III). We found the core problem in DD to be a vicious cycle of repeated visits at the same healthcare level, resulting in non-specific antibiotic treatment and failure to access specialized TB services.

To reduce the DD there is an urgent need for alternative means to monitor the epidemic at the local level. We found that a systematic registration of treatment delay in the TB program records of the Amhara Region of Ethiopia could be utilized to estimate the infectious pool of TB. By recording the treatment delay for new TB cases, retreatment cases and failures, and by estimating the number of undiagnosed cases, the total number of infectious days and, hence, an estimate of the infectious pool could be calculated (Paper IV).

Since DD was considerable in the high-incidence setting we wanted to compare to risk-groups in developed countries with a low-rate of TB (Paper V). Health care workers exposed to TB at three Norwegian hospitals as well as a non-exposed control group were tested with both TST and the INF-γ test T-SPOT.*TB*. Our data indicate that the frequency of latent TB in the total cohort of HCWs is 3% whereas the rate of transmission of TB to exposed individuals is around 2% and occurs through short time

exposure. Thus, the risk of TB transmission to health care workers following unprotected TB exposure in a hospital setting in Norway is low.

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III

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A systematic review of delay in the diagnosis and treatment of tuberculosis

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Abstract

Background: Early diagnosis and immediate initiation of treatment are essential for an effective tuberculosis (TB) control program. Delay in diagnosis is significant to both disease prognosis at the individual level and transmission within the community. Most transmissions occur between the onset of cough and initiation of treatment.

Methods: A systematic review of 58 studies addressing delay in diagnosis and treatment of TB was performed. We found different definitions of, for example, debut of symptoms, first appropriate health care provider, time to diagnosis, and start of treatment. Rather than excluding studies that failed to meet strict scientific criteria (like in a meta-analysis), we tried to extract the "solid findings" from all of them to arrive on a more global understanding of diagnostic delay in TB.

Results: The main factors associated with diagnostic delay included human immunodeficiency virus; coexistence of chronic cough and/or other lung diseases; negative sputum smear; extrapulmonary TB; rural residence; low access (geographical or sociopsychological barriers); initial visitation of a government low-level healthcare facility, private practitioner, or traditional healer; old age; poverty; female sex; alcoholism and substance abuse; history of immigration; low educational level; low awareness of TB; incomprehensive beliefs; self-treatment; and stigma.

Conclusion: The core problem in delay of diagnosis and treatment seemed to be a vicious cycle of repeated visits at the same healthcare level, resulting in nonspecific antibiotic treatment and failure to access specialized TB services. Once generation of a specific diagnosis was in reach, TB treatment was initiated within a reasonable period of time.

Background

Until the last part of the twentieth century, tuberculosis (TB) was a major cause of death in both developed and developing countries. Due to a range of factors such as the human immunodeficiency virus (HIV) epidemic, population growth, migration, socioeconomic changes, and broad spread of aggressive and resistant new strains such as the Beijing and W strains, a resurgence of TB has occurred, even in low endemic areas [1]. In 1993, the World Health Organization (WHO) declared a state of global emergency for TB due to the steady worldwide increase in the disease. Along with HIV and malaria, TB has been declared a global enemy. In 2005, 12 million new cases of tuberculosis were identified, a 58% increase

from the 7.5 million estimated cases in 1990, and it was estimated that in 2005 the disease caused 1.5 million deaths worldwide [2].

The goal of TB control programs is to arrest transmission within the community. Achieving this goal takes considerable time, because most individuals in endemic areas are already infected, constituting a reservoir that continuously contributes to the pool of infectious cases. An effective TB control program requires early diagnosis and immediate initiation of treatment. Delay in diagnosis is significant with regard to not only disease prognosis at the individual level but also transmission within the community and the reproductive rate of the TB epidemic [3,4]. Most transmissions occur between the appearance of cough and initiation of treatment. Madebo et al found that patients become more contagious as the delay progresses; the longest delays are associated with the highest bacillary numbers on sputum smears [5]. Because TB symptoms, particularly chronic cough with sputum, are so prevalent in most societies, early contact with health services causes delay rather than suspicion of TB. We found that in Ethiopia patients with TB symptoms contact an educated health worker on average after just 25% of the total delay period [6]. Thus, there is a fourfold-difference in the time of first contact and diagnosis.

Analysis of the factors leading to this delay between first contact and diagnosis is crucial to combatting the increasing TB epidemic. Although there are multiple studies of delayed TB diagnosis, no one has performed a systematic review.

Methods

Search strategy

We searched the following databases using the search terms and strategy described in Table 1: the Cochrane Infectious Diseases Group Specialized Register (February 2007); the Cochrane Central Register of Controlled Trials (CENTRAL) published in The Cochrane Library (February 2007); MEDLINE (1966 to February 2007); EMBASE (1974 to February 2007); and LILACS (1982 to February 2007). In addition, to identify unpublished and ongoing studies, we contacted individual researchers in the TB field as well as the WHO (2006) and the International Union Against Tuberculosis and Lung Disease (IUATLD, 2006).

Selection and analysis

Only observational studies were selected. All obtainable studies of patients receiving treatment for active pulmonary TB that recorded at least the median or mean total delay in diagnosis were included. The outcomes of interest were diagnostic delay from the debut of symptoms to the time of diagnosis or start of treatment. The titles and abstracts of the identified reports were used to exclude studies that clearly did not meet the inclusion criteria. For studies deemed potentially eligible for inclusion, we obtained the full paper. We screened the full articles of selected studies to confirm eligibility and resolved any disagreements by discussion. Our intent was not to exclude studies based on strict scientific criteria, or to perform a traditional quality assessment, but to make the studies as comparable as possible.

We analyzed the studies with the intent of identifying differences in approaches, rather than to define a gold standard. A primary aim was to describe the inevitable inaccuracy that arises from the use of different definitions of, for example, the debut of symptoms, first appropriate health care provider, time of diagnosis, and start of treatment.

Results

Search results

Our analysis revealed how complex it is to define diagnostic delay, and there were major differences between studies regarding inclusion and exclusion criteria, onset of symptoms, first contact and end of delay. First, the 58 studies used different inclusion criteria. Seventeen studies included all new TB cases, 11 included all pulmonary TB cases, 3 included all cases with a positive sputum smear, 24 included all new cases with a positive sputum spear, and for 3 studies data were not obtainable. Likewise, the study exclusion criteria differed. Some studies carefully excluded

Table 1: Search terms and strategy

Search set	CIDG SR	CENTRAL	MEDLINE	EMBASE	LILACS
1	Tuberculosis	Tuberculosis	Tuberculosis	Tuberculosis	Tuberculosis
2	Diagnostic delay	Diagnostic delay	Diagnostic delay	Diagnostic delay	Demora diagnostico*
3		Treatment delay	Treatment delay	Treatment delay	
4		Treatment seeking	Treatment seeking	Treatment seeking	
5		Case finding	Case finding	Case finding	
6		Help seeking	Help seeking	Help seeking	

CIDG SR: Cochrane Infectious Diseases Group Specialized Register
CENTRAL: Cochrane Central Register of Controlled Trials
*Demora diagnostico: Diagnostic delay

all cases with chronic underlying pulmonary conditions that could interfere with the patient's definition of symptom onset, but most did not. Some studies excluded visitors, mortal cases, and individuals with mental disturbances. The age-related exclusion criteria also varied: most studies excluded cases below the age of 16 years, some excluded cases below the age of 18 years, and a few included children of all ages. One study did not include patients who had undergone 2 or more months of treatment.

Definition of the onset of symptoms was also variable. Forty-nine studies defined onset as the debut of any symptom, 2 studies defined onset as debut of cough, and 1 study defined onset as debut of any pulmonary symptom. For 6 studies, a definition of symptom onset could not be obtained.

With regard to definition of the first contact, 34 studies defined the first contact as the first visit to a qualified healthcare provider. However, some of these studies included any allopathic ("western medicine") provider within the category of a qualified healthcare provider; others used the time of first contact with the national TB program in defining the end of patient delay. Eighteen studies defined the first contact as the time when the patient sought contact with any healthcare provider outside the household, including traditional practitioners. Four studies recorded both. Six studies did not provide any information with regard to definition of the first contact.

The studies also applied different definitions of the end of the delay. Seventeen studies defined the end of healthcare system delay as the time when a correct diagnosis was made (diagnostic delay), 20 studies defined it as the time the patient started treatment (treatment delay), and 14 studies distinctly recorded both. Data of this kind were unavailable for 7 studies.

Most studies defined the delay as a specific number of days, but several studies defined it as greater than a specific period of time (e.g. >60 days, > 90 days), or delay was defined as significantly longer in one group versus another group. A cut off point of 30 days to dichotomize into either delay on non delay was also commonly used.

Diagnostic delay

Table 2 lists the included studies in descending order according to the total diagnostic delay. Not surprisingly, the longest total delays (> 120 days) were reported for some high endemic countries, with the exception of the median 126-day delay reported by Lewis et al for East London [7]. Most of the studies, whether investigating low or high endemic countries, reported a total diagnostic delay within the range of 60–90 days (mean ± standard deviation: 72 days ± 28 days).

There was no consistent pattern with regard to the relative contributions of patients and healthcare providers to the diagnostic delay. The main delay was patient related in the studies in London (Lewis et al), Romania, Vietnam, Nigeria, South Africa, Australia (Queensland), Ethiopia (Addis Abeba), Korea, Somalia, Syria, Turkey (Istanbul, Okur et al), Japan (Chiba), Iraq, USA (New York), Yemen, and China (Shanghai). The main cause of delay was identified as the healthcare system in the studies of Tanzania, Ghana, Pakistan, Malaysia, Iran (WHO, nationwide), Botswana, New Zealand, Uganda, Ethiopia (Amhara), Italy, and China (Jianhu). Twelve studies reported a nearly equal contribution of patients and healthcare system to the diagnostic delay. The remaining studies did not record the relative importance of these two factors in the diagnostic delay.

Symptoms prior to diagnosis

Twenty-five studies recorded the frequency of symptoms reported by patients before diagnosis. The average frequencies of the following five cardinal symptoms were (number of studies listing the symptom in brackets): cough 85% (25), fever 65% (24), weight loss 62% (22), chest symptoms 50% (24), and haemoptysis 25% (22). Other symptoms less frequently reported: sputum 67% (5), fatigue 55% (8), and increased sweating 35% (10). All but two studies defined the onset of patient delay from the debut of any symptom [8,9], where the debut of cough defined the start.

Risk factors for prolonged diagnostic delay

The possible risk factors for diagnostic delay were heterogeneous (Table 3). The study conclusions were also heterogeneous; a risk factor for increased delay in some studies was a risk factor for decreased delay in other studies. Some factors were identified in numerous studies, while others were mentioned by only one study or a few studies. Following is a brief analysis of the factors, which are further elaborated in the "Discussion".

Clinical characteristics

The WHO study in Syria found HIV to be a risk factor for increased delay in diagnosis [10], while three other studies concluded the opposite [11-13]. Four studies found coexistence of chronic cough and/or other lung diseases be a risk factor for increased delay [12,14-16]; one study found the opposite [7]. Only a few studies included extrapulmonary TB, and as expected three found that patients with extrapulmonary TB experience longer delays than do patients with pulmonary TB [7,17,18]. Three studies found a negative sputum smear to be a risk factor for increased delay [12,19,20]; one study found the opposite (the WHO study in Egypt [15]). Six studies found alcoholism or substance abuse to be a risk factor for increased diagnostic delay [8,21-25]. Other health-related

Table 2: Median diagnostic delay for pulmonary TB patients in 58 studies

Country	Year	First author	DD Pat	DD HCP	DD total	Ref no.
Tanzania	2000	Wandwalo	15	120	136	[29]
United Kingdom	2003	Lewis	63	35	126	[7]
Burkina Faso	2006	Ouedraogo	*	*	120	[49]
Ethiopia	1999	Madebo	*	*	120	[5]
Ghana	1998	Lawn	28	56	120	[11]
Malawi	1988	Nkhoma	*	*	120	[54]
Thailand	1991	Tesana	*	*	120	[55]
Thailand	1993	Pungrassami	*	*	120	[45]
Romania	1989	Anastasatu	69♠	34♠	107♠	[33]
Iran	2002	Masjedi	13♠		93♠	[51]
Vietnam	1999	Long	54♠	29♠	93♠	[30]
Pakistan	2006	WHO	9	87	91	[27]
Malaysia	1994	Hooi	15	35	90	[21]
United States	2005	Golub	32	26	89	[22]
Iran	2006	WHO	24	42	88	[27]
Malaysia	1997	Liam	14	49	88	[36]
Botswana	1998	Steen	21	35	84	[26]
New Zealand	2000	Calder	7	49	84	[44]
Uganda	2005	Kiwuwa	14	63	84	[23]
Spain	2003	Altet Gomez	43♠	39♠	82♠	[24]
Ethiopia	2005	Yimer	15	61	80	[6]
Nepal	2001	Yamasaki-Nakagawa	23	29	79	[14]
Japan	1990	Niijima	*	*	78†	[46]
Mongolia	1996	Enkhbat	29	35	78	[35]
Nigeria	2004	Odusanya	56	7	70	[19]
South Africa	2001	Pronyk	28	7	70	[8]
Australia	2001	Ward	30	11	66	[20]
Thailand	2006	Rojpibulstit	31	20	66	[9]
China	2004	Bai	30	24	65	[28]
Italy	2006	Gagliotti	7	36	65	[38]
Spain	1996	Franco	23	32	64	[12]
Turkey	2004	Güneylioglu	18♠	13♠	64♠	[16]
Ethiopia	2002	Demissie	60	6	64	[18]
Norway	2006	Farah	28	33	63	[17]
United States	1998	Asch			60▲	[42]
India	2002	Rajeswari	20	23	60	[25]
Korea	1992	Mori	54♠	14♠	60♠	[31]
Peru	1996	Chavez	*	*	60	[53]
The Gambia	2001	Lienhardt	*	*	60	[32]
Zambia	2001	Needham	*	*	60	[10]
Cambodia	2006	Saly	*	10	58	[50]
Somalia	2006	WHO	53	7	58	[27]
Malawi	2000	Salaniponi	*	*	56	[47]
Syria	2006	WHO	31	15	55	[27]
China	2005	Xu	10	39	50	[37]
Turkey	2006	Okur	30	19	49	[34]
United Kingdom	2007	Rodger	*	*	49	[40]
Australia	1996	Pirkis	*	*	44	[15]
Taiwan	2005	Chiang	7	23	44	[56]
Egypt	2004	WHO	12	18	42	[27]
Japan	2002	Sasaki	21	7	42	[52]
Iraq	2004	WHO	31	2	36	[27]
United States	1999	Sherman	21	6	35	[39]
Yemen	2004	WHO	28	4	35	[27]
China	2006	Deng	19	5	31	[41]
France	1996	Gulbaran			30♠	[48]
Thailand	2001	Ngamvithayapong	11	8	*	[13]
Pakistan	2001	Sadiq			21€	[57]

Table 2: Median diagnostic delay for pulmonary TB patients in 58 studies *(Continued)*

The studies are listed in descending order of the median diagnostic delay.
DD Pat (Diagnostic Delay by the Patients): Time from debut of symptoms to first visit to health care provider
DD HCP (Diagnostic Delay by the Health Care Providers): Time from first visit to a HCP to the making of a proper diagnosis
DD Total (Total Diagnostic Delay): Time from debut of symptoms to the making of a proper diagnosis
* Data not obtainable
† Average calculated by the reviewers from separate numbers for female and male
♠ Mean
▲ 80% percentile
€ 77% percentile

Table 3: Risk factors for diagnostic delay

Risk factor	Positive association with risk	Negative association with risk
HIV	[10]	[11-13]
Coexistence of chronic cough and/or other lung diseases	[12, 14-16]	[7]
Negative sputum smear	[12, 19, 20]	[15]
Extrapulmonary TB	[7, 17, 18]	
Rural residence	[5, 11, 14, 16, 23, 25, 29-32]	
Low access to healthcare (geographical or socio-psychological barriers)	[6, 8, 10, 14, 18, 23, 25, 27-30, 34, 42, 47, 48, 50]	
Initial visit to government low-level healthcare facility	[5, 6, 9-11, 23, 26, 32-34]	[35]
Initial visit to traditional or unqualified practitioner	[9, 10, 14, 26-29, 32, 36, 37]	
Initial visit to private practitioner	[9, 10, 14, 26-29, 32, 36, 37]	
Initial visit to tertiary-level services/hospital	[11]	[13, 23, 38, 39]
Old age	[5, 12, 14-16, 19, 23, 24, 26, 38, 40, 41]	[18, 35]
Poverty	[7, 20, 21, 27, 28, 34, 37, 40, 41, 47, 48, 54, 56]	[18]
Female sex	[8, 10, 11, 14-16, 20, 22, 31, 33, 39, 40]	[5, 21, 23, 25]
Alcoholism or substance abuse	[8, 21-25]	
History of immigration	[8, 15, 17, 22, 38, 39, 42]	
Low educational level and/or low awareness and knowledge about TB	[9, 15-17, 20, 21, 23, 24, 27, 28, 31-33, 38, 39]	[13] (low educational level)
Other	Health-related reasons: Generally poor health [26] Smoking [14, 23] Coexistence of sexually transmitted diseases [26] Less severe and indifferent symptoms [27] No haemoptysis [16, 28] Socioeconomic factors: Married [5] Single [18, 26] Large family size [30] Farmer [5] White (vs. aboriginal) [20] Muslim [18] Belonging to an indigenous group [13] No insurance [13] Beliefs and attitudes: Beliefs about TB (not curable, caused by evil spirits, etc.) [8, 14, 27] Stigma [27] Self-treatment [6, 36, 42]	

The columns are identifying the applicable studies finding positive and negative association, respectively, with the risk factors

risk factors identified include generally poor health [26], smoking [14,23], coexistence of sexually transmitted diseases [26], less severe and indifferent symptoms [27], and absence of hemoptysis [16,28].

Socioeconomic factors

A range of studies found rural residence to be a risk factor for prolonged delay [5,11,14,16,23,25,29-32]. This risk factor seems to be closely linked to low access to healthcare and choice of settings in which to first seek healthcare

(see next section). Even among developing countries, access to healthcare varied. For example, in Ethiopia the public health service coverage was reported to be 50% [6], whereas in The Gambia 87% of the population was reported to have good access to healthcare [7,19,20,32]. The studies provided broad evidence that low access leads to prolonged delay in diagnosis [5,7,9,13,17,20,27-33].

Sociopsychological factors

Seeking government low-level health care facility first [5,6,9-11,23,26,32-34] (one study from Spain found the opposite [35]). Initially seeking a traditional or unqualified practitioner [9,10,14,26-29,32,36,37]. First seeking a private practitioner was a clear risk factor for diagnostic delay, independent of rural or urban residence [9-11,14,15,19,21,23,24,26,28,35]. Four studies concluded that seeking specialized services leads to a decreased diagnostic delay [13,23,38,39], while one study from the USA [11] found the opposite.

Sociodemographic factors

A range of studies found old age to be a risk factor for increased diagnostic delay [6,13,15-17,20,23,25,32,37-39], while two studies found the opposite [18,35]. Also, a number of studies concluded that females experience increased diagnostic delay [8,10,11,14-16,20,27,28,30,40,41]. However, a substantial number of studies made the opposite conclusion [5,21,23,25]. In addition, history of immigration or illegal residency seemed to be a risk factor in countries where this is actual [8,15,17,22,38,39,42].

Socioeconomic factors

Thirteen studies found low income and poverty to be a risk factor for diagnostic delay [3,7,17,23,24,31,34,38,39,43-46]. In a range of studies, low educational level and/or low awareness and knowledge about TB was listed as a risk factor for diagnostic delay [5,10,16-18,21,23,25,27-29,34,37,47,48]. Only one, from France [13], determined the opposite, finding that low educational level was linked to immigration and socially deprived groups where the health authorities focused on TB.

Other socioeconomic risk factors identified by 1–3 studies included being married [5], being single [18,26], large family size [30], being a farmer [5], being white (vs. aboriginal) [20], being a Muslim [18], belonging to an indigenous group [13], and not having insurance [13].

Beliefs and attitudes

Beliefs about TB (TB is incurable, caused by evil spirits, etc.) [8,14,27], stigma [27], and self-treatment [6,36,42] were identified as risk factors in 1–3 studies.

The vicious circle of repeated visits at the same level

A majority of the studies identified as the direct or underlying problem a vicious circle of repeated consultations with a multitude of healthcare providers without a correct diagnosis. Several papers list multiple visits at the same level, while others focus on multiple visits to the same physician. Three groups of healthcare providers were particularly identified as sources of this vicious circle: primary-level government health posts, who have limited diagnostic facilities and poorly trained personnel [5,6,9-11,23,26,32-34]; private practitioners with low awareness of TB [9-11,14,15,19,21,23,25,26,29,32]; and unqualified vendors, quacks, and traditional practitioners [9,10,14,26-29,32,36,37] In Burkina Faso for a patient seeking a health post or a private practitioner, the progression towards specialized services was poor; patients had repeated consultations at the same level, such that more than 94% of patients underwent repeated courses of non-specific antibiotics [49]. In Ghana, the health personnel at government health posts have poor training in diagnosing TB, and the specialized services of the NTP are over-centralized [11]. In a study in Malaysia, only 11% of patients received their diagnosis after the first consultation, and 45% received their first diagnosis after the third consultation [21]. Another study from Malaysia similarly indicates low awareness of the private practitioner as a key problem [36].

Discussion

The studies had different definitions of a range of variables, for example, debut of symptoms, first appropriate health care provider, time to diagnosis, and start of treatment, and they were not directly comparable or suited for a meta-analysis. Rather than excluding studies that failed to meet strict scientific criteria (like in a meta-analysis), we tried to extract the "solid findings" from all of them to arrive on a more global understanding of diagnostic delay in TB. Our detailed analysis demonstrates that the interacting factors affecting patients' health-seeking behavior and the availability of TB treatment can be categorized as involving either the patient or healthcare. Most factors influence both patient and health care delay, but some factors were more closely related to patient delay: alcohol or substance abuse, poverty, low access to health care facilities, rural residence, old age, belonging to an indigenous group and incomprehensive attitudes, beliefs and knowledge about TB. Other factors were evidently more linked to health care delay: coexistence of chronic cough and/or other lung diseases, having extrapulmonary or negative sputum smear TB, less severe and indifferent symptoms or absence of haemoptysis, poor health care infrastructure and seeking traditional and private practitioners first. As patients continue to go untreated, absent isolation, both components equally contribute to the infectious pool.

Our analysis revealed that the vicious circle of repeated visits at the same level is the core problem of diagnostic delay in most high endemic countries. TB is a rare disease, and more than 95% of patients with chronic cough seeking treatment at the level of primary healthcare do not have TB [43]. The delay in diagnosis on the part of health providers does not necessarily reflect inferior performance, but instead a lack of effective diagnostic tools and follow-up routines. Correct diagnosis requires both good training and available diagnostic facilities. The number of repetitions of this vicious cycle proved to be highly dependent on both the patient's beliefs and awareness of TB and the category of healthcare provider. In most countries, private practitioners are consulted first, and this pattern is significantly associated with a longer diagnostic delay. This pattern is also recognized as a source of bottleneck in low endemic countries. A New Zealand study observed that the first consulting doctor often did not perform an X-ray, ask about previous TB, or obtain a sputum smear – although the patients had classical symptoms [44]. The same observations were make by Ward et al in Australia [20]. Cough was treated symptomatically, and CXR were misdiagnosed.

A range of studies have shown that selection of a traditional practitioner for the first visit is associated with a prolonged delay in diagnosis [9,10,14,26-29,32,36,37]. In Yimer et al's study from Ethiopia, patients who first visited a qualified medical provider experienced a 21-day delay before initiation of treatment. Patients who first visited a traditional healthcare provider waited 15 days before first seeking healthcare, but the period from first visit to initiation of TB treatment was 4-fold greater [6]. These findings are supported by studies of The Gambia [32], Tanzania [29], and Penang [21].

Many studies describe a bottleneck in reaching the local unit of the NTP. Multiple studies observed poor access to the NTP as one of the main factors in delayed diagnosis [6,8,10,14,18,23,25,27-30,34,42,47,48,50]. In many high endemic countries, it appears crucial that a unit of the NTP is within a 1-day walking distance, as many patients have limited access to motorized vehicles. In addition to geographic distance, the studies identified several other barriers. One of the most important barriers is stigma [27]. Many patients were highly reluctant to visit the NTP facility, because it would mean disclosing to the public that they had TB. Even worse, in many countries, TB is so closely linked to HIV that patients fear they are revealing HIV status to their neighbors [37]. Also, many of the NTP personnel are unfriendly. Many patients feel deprived of both privacy and dignity. The widespread introduction of DOTS also means that patients must visit daily so that their consumption of medication can be directly observed; this is perceived as humiliating, time consuming, and a threat of a substantial loss of income [6,51,52]. A marginal farmer or a day laborer will often have to choose between treatment and placing food on the table for his family. Most of the studies from developing countries demonstrated a significant link between delayed diagnosis and poverty [7,20,21,27,28,34,37,40,47,48].

Most of the studies in our review also demonstrate the nonspecific nature of symptoms and the disease's first natural history as a core problem [15,18,23,31,50,53]. In the Gambia, TB is often misdiagnosed as malaria or viral infections [32].

Several studies also highlight the problem of self-treatment [6,36,42]. In the study in Pakistan, more than 50% of patients practiced self-treatment, and 42% first searched a pharmacy for their symptoms[27]. Many studies link this to the problem of stigma, in fact the unhappy triad of incomprehensive beliefs, low awareness, and stigma.

Conclusion

Our analysis is consistent with the findings of the WHO Eastern Mediterranean Region study. They concluded: "The private sector was the first choice for more than two-thirds of the patients. The main determinants of delay were: socio-demographic; economic; stigma; time to reach the health facility; seeking care from non-specialized individuals; and visiting more than one health care provider before diagnosis [3]." The core problem in delay of diagnosis and treatment seemed to be a vicious cycle of repeated visits at the same healthcare level, resulting in nonspecific antibiotic treatment and failure to access specialized TB services. Once generation of a specific diagnosis was in reach, TB treatment was initiated within a reasonable period of time.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

DGS, SY and GAB all contributed to develop the review protocol, DGS and SY performed data collection and analysis. DGS wrote the manuscript, and all the three authors edited and approved it.

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