Molecular epidemiology and drug resistance of *Mycobacterium tuberculosis* among HIV positive and HIV negative tuberculosis patients in Amhara region, Northwest Ethiopia

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Abstract

Tuberculosis is a major public health problem in Ethiopia. The aims of this study were (i) to investigate the recovery rate of *M. tuberculosis* from smear positive single morning sputum specimens subjected to long-term storage at -20°C, (ii) to assess the level and risk factors for first- and second-line anti-TB drug resistance, (iii) to evaluate the performance of the GenoType®MTBDRplus and GenoType®MTBDRsl assays for drug susceptibility testing compared to the BacT/ALERT 3D system as reference method, (iv) to analyze the frequency of gene mutations associated with resistance to isoniazid (INH), rifampicin (RMP) and ethambutol (EMB) among *M. tuberculosis* isolates, and (v) to study the population structure and transmission dynamics of *M. tuberculosis* isolates from patients in Amhara region, Northwest Ethiopia. The median specimen storage time was 132 days. Of 319 specimens, 90.0% were culture positive. The length of time of sputum storage had no significant effect on the recovery rate of *M. tuberculosis*. Of 260 *M. tuberculosis* isolates, 15.8% were resistant to at least one first-line drug, 5.0% were multidrug resistant (MDR) and 3.5% were resistant to all first-line drugs. Any resistance to INH, RMP, streptomycin (STM), EMB and pyrazinamide (PZA) was 13.8%, 5.8%, 10.0%, 7.3% and 4.6%, respectively. All isolates were susceptible to second-line drugs. The GenoType®MTBDRplus assay had a sensitivity of 92% and specificity of 99% to detect INH resistance, and 100% sensitivity and specificity to detect RMP resistance and MDR. The GenoType®MTBDRsl assay had a sensitivity of 42% and specificity of 100% to detect EMB resistance. According to the molecular methods, mutations conferring resistance to INH, RMP, or EMB were detected in 13.5%, 5.8%, and 3.1% of the isolates, respectively, while mutation conferring MDR was present in 5.0% of the isolates. Of 244 *M. tuberculosis* isolates, 59.0% were classified as known lineages; Dehli/CAS (38.9%), Haarlem (8.6%), Ural (3.3%), LAM (3.3%), TUR (2.0%), X-type (1.2%), S-type (0.8%), Beijing (0.4%) and Uganda II (0.4%) lineage. Interestingly, 31.6% of the isolates were grouped in to four previously undefined phylogenetic lineages and were named as Ethiopia_3 (13.1%), Ethiopia_1 (7.8%), Ethiopia_H37Rv like (7.0%) and Ethiopia_2 (3.7%) lineages. The remaining 9.4% of the isolates could not be assigned to the known or new lineages. Overall, 45.1% of the isolates were grouped in clusters, indicating high rate of recent transmission. Similarly, 66.7% of MDR strains were grouped in clusters.
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Abbreviations

AFB  Acid-fast bacilli
AIDS Acquired immunodeficiency syndrome
AM  Aminoglycosides
AMK Amikacin
ATS American Thoracic Society
BCG Bacillus Calmette-Guérin
CDC Centres for Disease Control and Prevention
CIP Ciprofloxacin
CM Cyclic peptides
CPB Cetylpyridinium bromide
CPC Cetylpyridinium Chloride
CPM Capreomycin
CS Cycloserine
DOTS Directly Observed Therapy-Short Course
DST Drug susceptibility testing
ELISA Enzyme-linked immunosorbent assay
EMB Ethambutol
ETO/ETH Ethionamide
FLD First-line anti-tuberculosis drug
FQs Fluoroquinolones
HGDI Hunter-Gaston discriminatory index
HIV Human immunodeficiency virus
IDSA Infectious Diseases Society of America
INH Isoniazid
KM Kanamycin
LFX Levofloxacin
L-J Lowenstein Jensen
MDR Multidrug resistance
MXF Moxifloxacin
MIRU-VNTR Mycobacterial interspersed repetitive unit-variable number tandem repeats
MST Minimum spanning tree
MTBC Mycobacterium tuberculosis complex
MUT Mutant
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>NALC</td>
<td>N-acetyl- L-cystine</td>
</tr>
<tr>
<td>NTM</td>
<td>Non tuberculosis mycobacteria</td>
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<tr>
<td>OFX</td>
<td>Ofloxacin</td>
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<tr>
<td>PAS</td>
<td>P-aminosalisylic acid</td>
</tr>
<tr>
<td>PZA</td>
<td>Pyrazinamid</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RMP</td>
<td>Rifampicin</td>
</tr>
<tr>
<td>RTI</td>
<td>Recent transmission index</td>
</tr>
<tr>
<td>SNNP</td>
<td>Southern nations, nationalities, and people’s</td>
</tr>
<tr>
<td>STM/SM</td>
<td>Streptomycin</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TTD</td>
<td>Time to detection</td>
</tr>
<tr>
<td>UPGMA</td>
<td>Unweighted pair group method with arithmetic averages</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>XDR</td>
<td>Extremely drug resistance</td>
</tr>
</tbody>
</table>
Introduction

Mycobacterium tuberculosis complex

The Mycobacterium tuberculosis complex (MTBC) comprised of closely related species including the human pathogens; M. tuberculosis, M. africanum and M. canettii and the animal adapted species; M. bovis (bovine), M. caprae (goats), M. pinnipedii (seals), and M. microti (rodents) (1-7). M. tuberculosis is the predominant cause of human tuberculosis (TB) worldwide but M. africanum and M. bovis remain important agents of human disease in certain geographical regions. Mycobacterium tuberculosis is a rod-shaped, non-motile, non-spore-forming, aerobic bacterium, classified as acid-fast bacilli, and has a unique cell wall structure crucial to its survival (8). The cell wall contains a considerable amount of fatty acid, mycolic acid, covalently attached to the underlying peptidoglycan-bound polysaccharide arabinogalactan, providing an extraordinary lipid barrier. This barrier is responsible for many of the medically challenging physiological characteristics of M. tuberculosis, including resistance to antibiotics and host defence mechanisms. The composition and quantity of the cell wall components also affect the bacteria’s virulence and growth rate (9).

Transmission and pathogenesis of M. tuberculosis

Mycobacterium tuberculosis spread by small airborne droplets generated during coughing, sneezing, talking, or singing of a person with pulmonary or laryngeal tuberculosis. The droplets are small enough in size (1-5 µm in diameter) to allow passage into the lower respiratory tract and can remain suspended in the air for several minutes to hours (10, 11). Droplets of a larger size are efficiently excluded from the lower respiratory tract by the physical barriers of the nasopharynx and upper respiratory tract (12). The transmission of M. tuberculosis from a TB patient to a contact person depends on (i) exposure duration; (ii) intensity of exposure; (iii) cough and sputum-related host factors; and (iv) M. tuberculosis strain-related virulence characteristics (13). The most important source of M. tuberculosis infection is a person with smear-positive pulmonary tuberculosis (14, 15).

Phases of M. tuberculosis infection and pathogenesis have been described previously (16-18). As shown in Figure 1, the first stage begins with inhalation of the tubercle bacilli. Once the bacilli have made their way into the lung, the bacilli are engulfed by alveolar phagocytic cells (macrophages and dendritic cells) and often destroyed. Tubercle bacilli which escape the initial intracellular destruction will replicate intracellularly, and the bacteria-laden phagocytic cells may cross the alveolar barrier to cause systemic dissemination. The intracellular replication and simultaneous dissemination of the pathogen to the pulmonary lymph nodes and to various other extrapulmonary sites occur prior to the development of the adaptive immune responses. In the vast majority of the infected individuals, an effective cell-mediated immune response develops 2–8 weeks after infection that stops further...
multiplication of the tubercle bacilli. The activated T lymphocytes, macrophages, and other immune cells form granulomas that wall off the growing necrotic tissue limiting further replication and spread of the tubercle bacilli. Most of the bacilli are killed in the granulomas, and disease progression is arrested. However, the bacilli are not completely eradicated in some individuals as *M. tuberculosis* escape killing by blunting the microbicidal mechanisms of phagocytic cells (such as phagosome-lysosome fusion, production of nitric oxide, and other reactive nitrogen intermediates). As a result, 5-10% of cases develop active disease (primary tuberculosis), while in the majority (90-95%) of cases the bacilli remain in a non-replicating (dormant/latent) state (latent tuberculosis infection), which is probably reflected by the *M. tuberculosis* DNA that can be detected using *in situ* PCR (shown in Figure 1 as blue intracytoplasmic material) in tissue. A subsequent defect in cell-mediated immunity may result in reactivation of dormant bacilli causing active disease many years after the infection (reactivation TB). Only approximately 10% of individuals with latent tuberculosis infection will develop the disease at some point of their life. For persons co-infected with HIV this risk increases to approximately 10% annually (19, 20). Progressive disease is characterized by weight loss, cavitations and fibrosis. Some of the cavities eventually open into the bronchi, which allow the bacilli to spread through the airways to other parts of the lung and the outside environment during coughing.

**Figure 1.** Phases of infection with *Mycobacterium tuberculosis* (18).
The global burden of tuberculosis

Tuberculosis remains one of the major public health problems worldwide. In 2009, WHO estimated 9.4 million incident cases of TB globally (equivalent to 137 per 100,000 population). The prevalence rate was estimated to be 200 per 100,000 population, which is equivalent to 14 million TB cases. The absolute number of cases continues to increase slightly from year to year, as slow reductions in incidence rates per capita continue to be outweighed by increases in population. Most of the estimated number of cases in 2009 occurred in Asia (55%) and Africa (30%). Smaller proportions of cases occurred in the Eastern Mediterranean region (7%), the European region (4%) and the region of the Americas (3%). The 22 high burden tuberculosis countries account for 81% of all estimated cases worldwide (21). Of the 9.4 million incident cases in 2009, an estimated 1.1 million (12%) were HIV-positive. Of which, approximately 80% were in the African region. Approximately 1.7 million people died of TB in 2009. Of which, 1.3 million deaths occurred among HIV-negative TB cases and 0.4 million deaths were among HIV-positive TB cases (21).

Tuberculosis in Ethiopia

According to the population census in 2007, Ethiopia has a total population of about 74 million. The second largest proportion of the country’s population is found in the Amhara region (23.3%), which is located in Northwest part of Ethiopia (22). According to the WHO’s global TB report, Ethiopia ranks seventh among the world’s 22 high-burden tuberculosis countries. The country had 314,267 TB cases in 2007, with an estimated incidence rate of 378 cases per 100,000 populations (23). The Ministry of Health hospital statistics data also showed that tuberculosis is one of the leading causes of morbidity, the fourth most common cause of hospital admission, and the second most common cause of hospital death in Ethiopia. TB mortality rate is estimated at 84 per 100,000 populations per year. The high prevalence of HIV infection, widespread poverty and overcrowding has created an environment which made tuberculosis a formidable threat in Ethiopia (24). The epidemiological analysis of tuberculosis in Ethiopia for the period of 2000-2007 has shown that the total number of new TB cases per year has increased from 83,334 to 126,809. An increasing trend was observed for smear positive TB cases from 26,459 to 38,040, smear negative TB cases from 30,333 to 43,500 and extrapulmonary TB cases from 26,542 to 45,269 during the eight years period (Fig. 2) (25). Similarly, another epidemiological study from 2000 to 2009 showed that the total number of new TB cases is generally increasing in Oromiya, Amhara and SNNP regions (26).
Figure 2. Notified cases of TB in Ethiopia in the last eight years (25).

EPTB=Extrapulmonary tuberculosis

Our previous report from Gondar, Northwest Ethiopia has shown that tuberculosis type among patients visited Gondar hospital between 2003 and 2008 was categorized as extrapulmonary in 1133 (28.3%) of cases, smear negative pulmonary tuberculosis in 2196 (54.9%) and smear positive pulmonary tuberculosis in 671 (16.8%) of cases. During the study period, high proportion of patients died (10.1%) or defaulted (18.3%) in the course of treatment (27).

The situation of HIV/AIDS in the Amhara region is one of the worst in the country. The prevalence was estimated at 2.8% in 2009 and 2.9% in 2010 in the region, which is higher than the prevalence of HIV/AIDS in the country estimated at 2.3% in 2009 and 2.4 in 2010 (28). In a previous study, TB-HIV co-infection rate, 52.1% was reported from Gondar hospital (29). Our previous study at Gondar hospital has also shown that the prevalence of HIV among blood donors was 3.5%. However, significantly declining trends of HIV prevalence was observed over the five years study period (2003-2007) (30). Moreover, our previous report from Gondar and Felege Hiwot hospitals showed encouraging levels of non-adherence (17.3%) and non-readiness (13.9%) of patients living with HIV/AIDS to highly active antiretroviral therapy (31).

Molecular Epidemiology of *M. tuberculosis*

Members of the MTBC are considered genetically monomorphic with a high level of genomic sequence similarity (> 99.95%), limited horizontal gene transfer, and a clonal population structure (32, 33). This apparent homogeneity led to the assumption that genetic diversity among MTBC strains would not be of clinical significance. However, recent data based on molecular genotyping methods
such as MIRU-VNTR (mycobacterial interspersed repetitive unit - variable number tandem repeat) typing, IS6110 RFLP and spoligotyping revealed a highly diverse population structure of *M. tuberculosis* with at least five major geographically-associated lineages including African (Uganda, Cameroon and S-type), Asian (Beijing and CAS), Latin American-Mediterranean (LAM), African-European populations (X-type, Ghana and Haarlem) and East African- Indian (EAI) lineages that can also be further subdivided into well-defined genotypes (34, 35).

Molecular epidemiological investigations have contributed significantly to the understanding of the epidemiology and control of tuberculosis by providing information on transmission dynamics (36), determining the importance of reactivation versus exogenous reinfection (37), investigating/confiming outbreaks (38), confirmation of laboratory cross contamination (39) and to identify the clonal spread of successful clones, including multi-drug-resistant ones (40). In addition, molecular markers can be used to evaluate host- and strain-specific risk factors and possible genotype-specific differences in phenotypes such as virulence and transmissibility (41-43).

**Anti-tuberculosis drugs and mechanisms of drug resistance in *M. tuberculosis***

Anti-tuberculosis drugs used for the treatment of TB are classified as first and second line drugs. First line drugs include ethambutol (EMB), isoniazid (INH), pyrazinamide (PZA), rifampicin (RMP) and streptomycin (STM). STM is no longer considered as a first line drug by ATS/IDSA/CDC because of high rates of resistance. Second line drugs include six classes of drugs: aminoglycosides e.g., amikacin (AMK) and kanamycin (KM); polypeptides e.g., capreomycin (CPM), viomycin and enviomyacin; fluoroquinolones e.g., ciprofloxacin (CIP), levofloxacin (LFX), ofloxacin (OFX) and moxifloxacin (MXF); thioamides e.g. ethionamide (ETO) and prothionamide; cycloserine (CS); and p-aminosalicylic acid (PAS). A drug may be classified as second-line instead of first-line for one of three possible reasons: it may be less effective than the first-line drugs; or, it may have higher toxic side-effects; or it may be unavailable in many developing countries (44).

According to a recently published review on the mechanisms of drug resistance in *M. tuberculosis* (45), genetic resistance to anti-tuberculosis drug is due to spontaneous chromosomal mutations at a frequency of $10^{-6}$ to $10^{-8}$ mycobacterial replications. Mobile genetic elements such as plasmids and transposons, which are known to mediate drug resistance in various bacterial species, do not do so in *M. tuberculosis*. Mutations resulting in drug resistance are unlinked, thus the probability of developing bacillary resistance to three drugs used simultaneously is virtually non-existent. However, amplification of the afore-mentioned genetic mutation through human mistakes results in clinically
Introduction

drug resistant TB. Human mistakes include monotherapy due to irregular drug supply, inappropriate prescription and, most importantly, poor patient compliance (46). Subsequent transmission of resistant strains from the index patient to others aggravates the problem. Alternative mechanisms such as active efflux pumping and decreased cell wall permeability to drugs are likely to be important for several drugs especially in phenotypically resistant isolates in which no mutation is detected in the target genes (47-52).

Table 1. Mechanisms of drug resistance in *M. tuberculosis* (45)

<table>
<thead>
<tr>
<th>Drug (year of discovery)</th>
<th>MIC g/ml</th>
<th>Gene(s) involved in resistance</th>
<th>Gene function</th>
<th>Role</th>
<th>Mechanism of action</th>
<th>Mutation frequency %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid (1952)</td>
<td>0.02–0.2</td>
<td>katG, inhA</td>
<td>Catalase-peroxidase, Enoyl ACP reductase</td>
<td>Pro-drug conversion, Drug target</td>
<td>Inhibition of mycolic acid biosynthesis and other multiple effects</td>
<td>50–95</td>
</tr>
<tr>
<td>Rifampicin (1966)</td>
<td>0.05–1</td>
<td>rpoB</td>
<td>β subunit of RNA polymerase</td>
<td>Drug target</td>
<td>Inhibition of RNA synthesis</td>
<td>95</td>
</tr>
<tr>
<td>Pyrazinamide (1952)</td>
<td>16–50</td>
<td>pncA</td>
<td>Nicotinamidase/pyrazinamidase</td>
<td>Pro-drug conversion, Drug target</td>
<td>Depletion of membrane energy Inhibition of arabinogalactan synthesis</td>
<td>72–97</td>
</tr>
<tr>
<td>Ethambutol (1961)</td>
<td>1–5</td>
<td>embB</td>
<td>Arabinosyl transferase</td>
<td>Drug target</td>
<td>Inhibition of protein synthesis</td>
<td>47–65</td>
</tr>
<tr>
<td>Streptomycin (1944)</td>
<td>2–8</td>
<td>rpsL, rrs, gidB</td>
<td>S12 ribosomal protein, 16S rRNA, rRNA methyltransferase (G527 in 530 loop)</td>
<td>Drug target, Drug target, Drug target</td>
<td>Inhibition of protein synthesis</td>
<td>52–59, 8–21, ?</td>
</tr>
<tr>
<td>Amikacin/kanamycin (1957)</td>
<td>2–4</td>
<td>tlyA</td>
<td>2'-O-methyltransferase</td>
<td>Drug target</td>
<td>Inhibition of protein synthesis</td>
<td>76</td>
</tr>
<tr>
<td>Capreomycin (1960)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quinolones (1963)</td>
<td>0.5–2.5</td>
<td>gyrA, gyrB</td>
<td>DNA gyrase subunit A, DNA gyrase subunit B</td>
<td>Drug target</td>
<td>Inhibition of DNA gyrase</td>
<td>75–94</td>
</tr>
<tr>
<td>Ethionamide (1956)</td>
<td>2.5–10</td>
<td>etaA, ethA, inhA</td>
<td>Flavin monooxygenase, Drug target</td>
<td>Drug target</td>
<td>Inhibition of mycolic acid synthesis</td>
<td>37</td>
</tr>
<tr>
<td>PAS (1946)</td>
<td>1–8</td>
<td>thyA</td>
<td>Thymidylate synthase, Drug activation?</td>
<td>Drug target</td>
<td>Inhibition of folic acid and iron metabolism?</td>
<td>36</td>
</tr>
</tbody>
</table>

MIC = minimum inhibitory concentration; ACP = acyl carrier protein; PAS = para-aminosalicylic acid.

According to the WHO report, the overall proportion of multidrug resistant tuberculosis (MDR-TB) was 5.3%, ranging from 0% to 35% of reported TB cases globally (53). The number of countries reporting at least one case of extremely drug-resistant tuberculosis (XDR-TB) had risen to 58 by January 2010, although this rise is probably a result of increased efforts to identify XDR tuberculosis rather than an increase in prevalence or distribution of the disease (54).
Drug resistant TB in Ethiopia

In Ethiopia, there have been reports on drug resistance of TB, mainly from the capital city, Addis Ababa. A report on anti-TB drug resistance, probably the first in the country among new TB patients of Addis Ababa was reported in 1984, this report showed a drug resistance rate of 15% for isoniazid, 5% for streptomycin and 5% for both isoniazid and streptomycin. There was no rifampicin resistance reported and hence no MDR TB (55). The first MDR TB prevalence among new cases was reported in 1986 to be 1% (56). In 1998, a study which included all health centres and hospitals of Addis Ababa reported 0.6% MDR-TB prevalence (57). In 2001, a report from Addis Ababa showed the level of MDR-TB was nearly 2.7% among new cases and 5.3% for new and re-treatment cases combined (58). Later on, a study performed in 2003 on re-treatment patients of St. Peter’s hospital in Addis Ababa showed 26% MDR (59).

The countrywide anti-TB drug resistance survey conducted in 2005 showed that the prevalence of MDR-TB was 1.6% and 11.8% among new and previously treated TB cases, respectively (52). Moreover, according to WHO report in 2008, about 5825 MDR-TB cases (4964 among newly diagnosed and 861 among previously treated TB cases) were estimated to have occurred in 2006 in Ethiopia (54). Consequently, the Ethiopian government has identified MDR-TB as one of priority public health problem and started treatment program for MDR-TB patients. Treatment of MDR-TB patients started in 2009 at St. Peter hospital, Addis Ababa and in 2010 at Gondar University hospital, in the Amhara region. Since then, the number of patients on treatment has been increasing steadily, but still only a fraction of the estimated Ethiopians with MDR-TB has access to the treatment (24). In Ethiopia, the treatment regimens for category I and category II (retreatment regimen) tuberculosis cases are 2 months (RMP-INH-EMB-PZA) plus 4 months (RMP-INH) and 2 months STM (RMP-INH-EMB-PZA) plus 1 month (RMP-INH-EMB-PZA) plus 5 months (EMB$_3$ (RMP-INH)$_3$), respectively (25). The standard treatment regimen for MDR-TB is 6 months (EMB-PZA-KM (AMK)–LFX–ETO-CS) plus 12 months (EMB-PZA-LFX–ETO–CS) (24).

The rationale for the study

The objectives of a TB control program are to reduce morbidity, mortality, transmission of TB and emergence of drug resistance. Well-planned TB control programs may lead to achieve these goals (60, 61). However, the TB control program in Ethiopia suffers from shortage of information necessary for proper planning and evidence-based decision making. Therefore, this study was done to fill these information gaps.
Introduction

Despite the high prevalence of tuberculosis in the country, culture and drug susceptibility testing of *M. tuberculosis* is being done only in the national reference laboratory, in the capital city, Addis Ababa. The successful isolation of *M. tuberculosis* from remote settings requires proper collection, storage and transportation of sputum specimens to TB laboratories (62-64). To ensure high yield of positive cultures, WHO recommends that two sputum specimens be collected from remote areas and transported to reference laboratories without delay (63). There may be a substantial cost associated with handling, transportation and processing of two sputum specimens from each patient. In addition, sputum specimen preservatives such as 0.6% cetylpyridinium bromide (CPB) or 1% cetylpyridinium chloride (CPC) are recommended when delay is unavoidable. CPC, the most commonly used preservative for the storage and transport of sputum specimens keeps the tubercle bacilli viable only for up to 8 days (65-68). Moreover, previous studies have shown the negative effect of CPC on microscopic examination and culture, including: (i) significant reduction in the positivity of AFB with Ziehl-Neelsen staining (69, 70) and (ii) inhibition of mycobacterial growth, when inoculated in culture media like Middlebrook 7H9 and 7H10 (67) and Bactec MGIT 960 system (71). A simple and inexpensive method for sputum storage that preserves the viability of tubercle bacilli for long periods will be useful for epidemiological and drug resistance studies from remote settings. Therefore, the present study was undertaken to investigate the recovery rate of *M. tuberculosis* from smear positive single morning sputum specimens subjected to long term storage at -20°C without addition of chemical preservatives (Publication I).

In most high-burden TB countries including Ethiopia, drug resistant TB is only diagnosed after prolonged treatment with first-line TB drugs and clinical recognition that treatment has failed. Treatment of drug-resistant TB with standard first-line drugs, instead of a regimen designed according to the resistance pattern has several potential adverse consequences: patients remain on inadequate treatment longer, increasing the risk of treatment failure or death; selection of drug resistant strains and patients remain infectious, promoting transmission to close contacts (72). In order to optimize standard anti-tuberculosis drug therapy and to increase the success of control programs, it is important to know drug resistance pattern in a country (73). However, only limited data on drug resistant TB are available in Ethiopia. Thus, there is a great need to study the extent of the drug resistant tuberculosis in Ethiopia in order to develop effective control strategies. Therefore, this study aimed to assess the level and risk factors for first and second-line drug resistance among new untreated and previously treated smear positive tuberculosis patients in Northwest Ethiopia (Publication II).
Introduction

For proper treatment and control of tuberculosis, the WHO is recommending countries to expand their capacity for culture based drug-susceptibility testing (DST) and consider new molecular-based assays for diagnosing drug resistance (74, 75). Molecular methods of drug resistance testing, based on the identification of gene mutations associated with drug resistance, like GeneXpert MTB/RIF assay, offer an effective tool for determining drug resistance because of their high sensitivity, specificity and speed (76). New molecular methods that have been developed to detect drug resistance include the GenoType®MTBDRplus for detection of isoniazid and rifampicin resistance and the GenoType®MTBDRsl for detection of resistance against ethambutol, fluoroquinolones (e.g. ofloxacin and moxifloxacin), and aminoglycosides/cyclic peptides (e.g. capreomycin, viomycin/kanamycin and amikacin) (Hain Lifescience, Nehren, Germany).

In Ethiopia, five regional laboratories are being rebuilt and equipped to perform culture and drug susceptibility testing using methods including GenoType®MTBDRplus and GenoType®MTBDRsl assays. The GenoType®MTBDRplus and GenoType®MTBDRsl assays have been studied in several laboratories of other countries. However, there is a wide variation in circulating M. tuberculosis strains worldwide (77, 78), and false negative results reported due to unique genetic mutations, affecting the performance of molecular assays (79-85). Therefore, analysis of gene mutations associated with resistance to anti-tuberculosis drugs and assessment of the performance of molecular methods for drug resistance testing in different settings are needed to ensure acceptable performance of the assays. So far, there was no report on the frequency of gene mutations associated with resistance to INH, RMP and EMB and the applicability of these molecular assays for M. tuberculosis isolates from Ethiopia. In this study, we evaluated the performance of the GenoType®MTBDRplus for detection of resistance to INH, RMP and MDR and GenoType®MTBDRsl assay for detection of EMB resistance compared to the automated, culture-based BacT/ALERT 3D system and analyzed the frequency of gene mutations associated with resistance to INH, RMP and EMB among M. tuberculosis isolates from Amhara region, Northwest Ethiopia (Publication III).

Recent advances in molecular technology such as mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) 24-loci typing and spoligotyping methods provide a powerful tool to analyze M. tuberculosis genotypes and transmission dynamics, which should be valuable for development of effective tuberculosis control policy. In Ethiopia limited molecular epidemiological studies have been done so far only for isolates from patients in the capital city, Addis Ababa (86-88). These studies were limited by small sample size, incomplete epidemiological information and use of a genotyping method with low strain discriminatory capacity. Based on IS6110 fingerprinting or
spoligotyping, a variety of strains were not classifiable into phylogenetic lineages or clonal complexes since the genotyping information was not informative due to homoplasy (89, 90). Therefore, the presence of particular genotypes and clustering might simply be overlooked. Because these questions have paramount scientific and public health importance, further studies addressing these questions using more appropriate methods are urgently needed. The new genotyping technique based on MIRU-VNTR 24-loci typing allows the simultaneous high-resolution discrimination of clinical isolates for epidemiological studies and a valid phylogenetic strain classification (91). In this study, we investigate the population structure and transmission dynamics of *M. tuberculosis* strains isolated from patients living in Amhara region using MIRU-VNTR 24-loci and spoligotyping methods (Publication IV).
Objectives

General objective
The overall aim of this study was to investigate a simple and inexpensive sputum storage method, to document the patterns of drug resistance and gene mutations conferring resistance to anti-TB drugs, to evaluate the performance of two molecular based drug susceptibility testing methods and to analyze the population structure and transmission dynamics of *Mycobacterium tuberculosis* among HIV positive and HIV negative tuberculosis patients in Amhara region, Northwest Ethiopia.

Specific objectives

1. To investigate the recovery rate of *M. tuberculosis* from smear positive single morning sputum specimens subjected to long term storage at -20°C without addition of chemical preservatives (Publication I).

2. To assess the level and risk factors for first and second line anti-TB drug resistance among new untreated and previously treated tuberculosis patients in Amhara region (Publication II).

3. To evaluate the performance of the GenoType®MTBDR plus for detection of resistance to INH, RMP and MDR and GenoType®MTBDRsl assay for detection of EMB resistance among *M. tuberculosis* isolates from patients in Amhara region compared to the BacT/ALERT 3D system (Publication III).

4. To analyze the frequency of gene mutations associated with resistance to INH, RMP and EMB among *M. tuberculosis* isolates from patients in Amhara region (Publication III).

5. To study the population structure and transmission dynamics of *Mycobacterium tuberculosis* among tuberculosis patients living in Amhara region (Publication IV).
Rate of Recovery of *Mycobacterium tuberculosis* from Frozen Acid-Fast-Bacillus Smear-Positive Sputum Samples Subjected to Long-Term Storage in Northwest Ethiopia

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Tuberculosis is a major public health problem in Ethiopia. The diagnosis and treatment of drug-resistant tuberculosis remain a challenge in the country. This study aimed to assess whether single morning sputum samples could be stored at −20°C for extended periods of time at remote settings and then transported and successfully cultured for *Mycobacterium tuberculosis*. Single morning sputum samples were collected from all smear-positive tuberculosis patients diagnosed at Gondar Hospital, Gondar Health Center, Metemma Hospital, Bahir Dar Hospital, and Debre Markos Hospital in Northwest Ethiopia between March and July 2009. Specimens were stored at the study sites and sent to the mycobacteriology laboratory of the University Hospital, Leipzig, Germany, where specimens were processed and inoculated into the BacT/Alert 3D system and Lowenstein-Jensen and Gottesacker media. Ice packs were added in the package of the specimens during transport. A total of 319 patients were enrolled in this study. The median specimen storage time was 132 days (range, 16 to 180 days). Of all specimens, 283 (88.7%) were culture positive by any of the three culturing systems. *M. tuberculosis* isolates from four contaminated specimens in all culturing systems were successfully isolated on Middlebrook 7H10 agar; thereby, the recovery rate increased to 287 (90.0%). The length of time of sputum storage had no significant effect on the rate of recovery of *M. tuberculosis* in all culturing systems. In conclusion, single morning sputum specimens collected at remote settings stored at −20°C for long periods of time without the addition of preservatives can yield a high recovery rate. These findings suggest a simple and cost-effective alternative method of sputum storage for epidemiological and drug resistance studies in low-resource countries.

Tuberculosis (TB) remains a leading infectious cause of morbidity and mortality worldwide. In 2007, the World Health Organization (WHO) estimated 9.27 million new cases of TB, with 1.3 million deaths globally (22). Africa still carries a disproportionate burden of global TB cases, 29% of all TB cases (1). Ethiopia ranks seventh among the world’s 22 high-burden tuberculosis countries. The country had 314,267 TB cases in 2007, with an estimated incidence rate of 378 cases per 100,000 people (22).

The nationwide anti-TB drug resistance survey in 2005 showed that the prevalences of multidrug-resistant TB (MDR-TB) were 1.6% among new cases and 11.8% among previously treated TB cases (21). However, the isolation and drug resistance testing of *Mycobacterium tuberculosis* remain a challenge in Ethiopia; so far, there are only two laboratories in the capital city, Addis Ababa, that perform culture and drug sensitivity testing for *M. tuberculosis*. The successful isolation of *M. tuberculosis* from remote settings requires proper collection, storage, and transportation of sputum specimens to TB laboratories (4, 19, 20).

The WHO recommends that two sputum specimens be collected from remote areas and transported to reference laboratories without delay (19). There may be a substantial cost associated with the handling, transportation, and processing of two sputum specimens from each patient without delay. In addition, sputum specimen preservatives, such as 0.6% cetylpyridinium bromide (CPB) or 1% cetylpyridinium chloride (CPC), are recommended when a delay is unavoidable. CPC is known to effectively liquefy and decontaminate sputum samples and keep tubercle bacilli viable for up to 8 days; it is still the most commonly used preservative for the storage and transport of sputum specimens (12, 15, 17, 18).

Once CPB or CPC has been added to a specimen, refrigeration as a storage method is no longer possible (19). Moreover, once preserved specimens have reached the culture laboratory, a centrifugation step without refrigeration is necessary to remove the preservative prior to culture (12). Previous studies have also shown the negative effects of CPC on microscopic examination and culture, including (i) a significant reduction in the positivity of acid-fast bacilli (AFB) with Ziehl-Neelsen staining (14, 16) and (ii) the inhibition of mycobacterial growth, especially when inoculated into culture media, including Middlebrook 7H9 and 7H10 media (17), and the Bactec MGIT 960 system (13). Palomino et al. also recommended that sputum samples should be transported rapidly to the reference laboratory to avoid overgrowth by other microorganisms; when
the transport or processing of the sputum is delayed, specimens should be stored for no more than 5 days at 4°C until they are transported or processed for culture (8).

A simple inexpensive method for sputum storage and transport that preserves the viability of tubercle bacilli for long periods of time will be useful for epidemiological and drug resistance studies in remote settings. Therefore, the present study was undertaken to assess the rate of recovery of M. tuberculosis from single morning sputum specimens subjected to long-term storage at -20°C without the addition of chemical preservatives.

MATERIALS AND METHODS

Study design, area, and study period. All smear-positive pulmonary tuberculosis (PTB) patients diagnosed at Gondar Hospital, Gondar Health Center, Meckuma Hospital, Bahir Dar Hospital, and Debre Markos Hospital in Northwest Ethiopia between 1 March 2009 and 15 July 2009 were included in this study. The diagnosis of smear-positive PTB was based on national guidelines for the microscopic examination of tuberculous (7); direct smears were prepared from three sputum specimens. The first specimen was collected on the spot when the patient presented at the diagnostic center. The patient was then given another sputum container and instructed to collect an early-morning specimen on the next day and return it to the clinic. A third specimen was collected when the next-day specimen was delivered to the laboratory. Smears were stained by a Zeih-Neelsen staining technique for microscopic examination without grading smear positivity. Only diagnosed, informed consent was obtained from the study subjects, and the single morning sputum sample was collected for storage and transport to the microbiology laboratory in Gondar for culturing. Instructed smear clearing was obtained from the research and publication consent of Gondar University, Gondar, Ethiopia.

Storage and transport of sputum specimens. The specimens were kept at 26°C without the addition of chemical preservatives, in the TB laboratory (uncontaminated) until they were prepared for transport to the mycobacteriology laboratory at the Institute of Medical Microbiology and Epidemiology of Infectious Diseases, University Hospital Leipzig. The preparation and packaging of the specimen complied with International Air Transport Association (IATA) regulations (http://www.iata.org/audi/eng.htm). No decontamination procedure was performed prior to storage or transportation. Specimens were transported in one batch after the data collection period. Ice packs were added to the package of the specimens during transport, and specimens were stored at -20°C after arrival at the mycobacteriology laboratory in Leipzig until they were processed for culture.

Specimen processing and culturing methods. Specimens were processed for culture from 27 July to 6 September 2009 according to Deutsches Institut für Normung (DIN) recommendations for the detection of mycobacteria by culture methods shown in Table 2. In brief, sputum samples were transferred into centrifuge tubes with a capacity of 50 ml, distilled water was added up to 10 ml, and the same volume (10 ml) of a Na-acetate-lysine-NaOH solution (4% NaOH, 20% sodium citrate, and 0.5% NaCl) was added and incubated at room temperature on the shaker for 20 min and then neutralized with phosphoric buffer (pH 6.8) and concentrated by centrifugation (3,300 × g for 20 min). From the spumum concentrates, 0.5 ml was used for inoculation into Bact/Alert MF bottles supplied with the results of the manufacturer’s instruction manual. Bact/Alert MF bottles (Bio-Mérieux SA, France), 0.2 ml was inoculated into Lowenstein-Jensen (L-J) medium, and the same amount was inoculated into Gutstock medium. Both L-J and Gutstock media (Arcueil-ENCIIT GmbH, Wym, Germany) were supplemented with polymyxin B (200,000 IU/liter), amphotericin B (10 µg/ml), cefoxitin (250 µg/ml), and trimethoprim (10 µg/ml) (PACT). Spumum concentrates were also used to prepare smears for the grading of the smear positivity of the sputum. The remaining spumum concentrates were stored at -80°C for further investigation by PCR.

Bact/Alert MF bottles were loaded and incubated into the Bact/Alert 3D system incubator chamber for a maximum of 56 days at 36°C. The L-J and Gutstock plates were read twice on the first week and weekly thereafter for a total of 7 weeks of incubation at 36°C. Microscopy of spumum concentrates was performed by using an auramine-rodaminrine stain, and the results were recorded by using standardized grading scales (3). Positive bottles and plates were sampled for solid-fast bacillus (AFB) staining by the Kinyoun staining method and inoculated onto a blood agar plate (overnight incubation) to confirm culture purity. Concentrated fluid cultures were inoculated onto Middlebrook 7H10 agar (BD Diagnostics, Heidelberg, Germany) without decontamination for the isolation of pure mycobacterial colonies. Samples that were culture negative by both solid and fluid culture media and samples that were contaminated in fluid cultures but negative in solid media were analyzed by PCR (Cobas AmpliCycler analyzer; Roche Diagnostics, Indianapolis, IN) for M. tuberculosis complex organisms.

Identification of mycobacteria. Isolates with the typical morphology of members of the M. tuberculosis complex were identified by DNA hybridization technology with microcellulose strips (GenTyp e MTBC: Hain Diagnostika, Nehren, Germany) according to the manufacturer’s instructions. The procedure involved the isolation of DNA from cultured material, multiplex amplification with biotinylated primers, and reverse hybrization of the single-stranded, biotin-labeled amplimers onto membrane-bound probes. The resulting binding pattern indicates the species of the M. tuberculosis complex. M. tuberculosis was differentiated from Mycobacterium canettii based on the colony morphology of the isolates on solid media.

Statistical analysis. All laboratory data were entered, cleared, and analyzed by using SPSS, version 13, statistical package software (SPSS Inc., Chicago, IL). The effects of sputum storage time, smear positivity, and sputum volume on the rate of recovery and time to detection of M. tuberculosis growth were analyzed in this study. The measurement of time to detection was of a nonnormal distribution, and we have therefore reported summary statistics of medians and ranges. We used a linear regression model to analyze predictors of a successful culture. The differences in the rates of recovery of M. tuberculosis among different culture systems were determined by the McNemar modification of the chi-square test. The comparison of the median times for a positive culture among fluid and solid culture systems was made by the Wilcoxon matched-pairs test. One-way analysis of variance (ANOVA) was used for trend analyses across ordered groups. P values of less than 0.02 were considered statistically significant.

RESULTS

A total of 319 smear-positive pulmonary tuberculosis patients were enrolled in this study. The median specimen storage time from collection until processing for culture was 122 days (range, 16 to 180 days). The specimen transportation time from the hospitals in Ethiopia to the mycobacteriology laboratory in Germany was 6 days. All samples did arrive thawed at the mycobacteriology laboratory in Germany. Of all specimens, 97/319 (30.4%) had a small sputum volume (< 2 ml); some specimens had as little as 300 µl. The majority, 222/319 (69.6%) specimens, had a large volume (2 to 5 ml). Additionally, 29/319 (9.1%) specimens had low-level smear positivity (scores of doubtful and ++), and 290/319 (90.9%) had a high level of smear positivity, with scores of ++ and above.

Of all sputum specimens, 283/319 (88.7%) were culture positive by any of the three culture methods, 4/319 (1.3%) were contaminated in all cultures, 26/319 (8.1%) failed to grow in all systems, and 6/319 (1.9%) were found to be contaminated in the Bact/Alert 3D system and failed to grow in both L-J and Gottsacker media (Table 1). M. tuberculosis isolates from four contaminated specimens in all cultures were successfully isolated on Middlebrook 7H110 agar medium, thereby increasing the recovery rate to 287/319 specimens (90.0%). PCR was performed for 26 specimens that were negative by all culture methods and 6 specimens that were contaminated in fluid cultures and failed to grow in solid media. The PCR results showed that 27/32 (84.4%) specimens were positive for organisms of the M. tuberculosis complex, 3/32 (9.4%) were inhibited, and 2/32 (6.2%) were negative. All positive cultures were identified as Mycobacterium tuberculosis. No nontuberculosis mycobacterium (NTM) was isolated from any study subject.

The differences in the rates of recovery of M. tuberculosis among the three culture methods were not statistically significant. The contamination rate was found to be high, 42/319 specimens (13.2%), in the Bact/Alert 3D system, compared to
TABLE 1. Rates of recovery of M. tuberculosis by the BacT/Alert 3D system, L-J medium, and Gottschalk medium during primary culture

<table>
<thead>
<tr>
<th>Culture result</th>
<th>BacT/Alert 3D system (n = 319)</th>
<th>L-J medium (n = 319)</th>
<th>Gottschalk medium (n = 319)</th>
<th>L-J and Gottschalk medium (n = 319)</th>
<th>BacT/Alert 3D system and L-J and Gottschalk medium (n = 319)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive (M. tuberculosis)</td>
<td>251 (78.7)</td>
<td>249 (78.0)</td>
<td>256 (74.7)</td>
<td>265 (83.1)</td>
<td>283 (88.7)</td>
</tr>
<tr>
<td>Contamination</td>
<td>42 (13.2)</td>
<td>21 (6.6)</td>
<td>16 (5.0)</td>
<td>10 (3.1)</td>
<td>4 (1.3)</td>
</tr>
<tr>
<td>Negative</td>
<td>26 (8.1)</td>
<td>49 (15.4)</td>
<td>67 (21.3)</td>
<td>44 (13.8)</td>
<td>6 (1.9)</td>
</tr>
<tr>
<td>Contamination in BacT/Alert 3D and negative in solid media</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* At least one positive.
* All media were contaminated.
* All media were negative.
* P < 0.001 for the BacT/Alert 3D system versus L-J medium, P = 0.002 for the BacT/Alert 3D system versus Gottschalk medium, and P = 0.007 for L-J medium versus Gottschalk medium (determined by the McNemar test for differences).

21/319 (6.6%) in L-J medium and 16/319 (5.0%) in Gottschalk medium. However, the higher contamination rate was compensated for by a higher rate of recovery of M. tuberculosis in fluid culture.

The length of time of sputum storage had no significant effect on the proportion of positive culture results for all culture methods. Higher-level smear positivity was more predictive of a positive culture result (P < 0.001) for all culture methods. The rate of recovery of M. tuberculosis from samples with low-level smear positivity was 4/5 specimens (80%) for doubtful smear-positive samples and 12/24 (50%) for smear-positive samples with a score of 1+. The rates of recovery of M. tuberculosis from samples with high-level smear positivity were 37/44 specimens (84.1%) with a score of 2+, 81/89 (91.0%) with a score of 3+, and 149/157 (94.0%) with a score of 4+ for smear-positive samples by all culture methods. The larger volume of specimen was more predictive of a positive culture in L-J medium (P = 0.023) and Gottschalk medium (P < 0.001) (Table 2).

The median time required for the detection of M. tuberculosis growth by each culturing method is summarized in Table 3. The BacT/Alert 3D system detected the growth of M. tuberculosis earlier (median, 14 days; range, 6 to 48 days) than did L-J and Gottschalk media (median, 19 days; range, 10 to 60 days) (both P < 0.001). The sputum storage time had no significant effect on the time to positive culture by L-J medium and Gottschalk medium, but the longer sputum storage time was significantly associated (P < 0.001) with a longer time to positivity by the BacT/Alert 3D system. Sputum smear positivity was inversely related to the time to positivity of the BacT/Alert 3D system (P < 0.001); however, it was not statistically significant for L-J medium and Gottschalk medium. The sputum volume was inversely related to the time to positive culture by the BacT/Alert system (P < 0.001) and L-J medium (P = 0.008).

**DISCUSSION**

In the present study, the rate of recovery of M. tuberculosis from single morning sputum specimens subjected to long-term storage (median, 132 days; range, 16 to 180 days) at −20°C without the addition of chemical preservatives was very high.

TABLE 2. Effects of smear positivity after decontamination, storage time of sputum, and sputum volume on the rate of recovery

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Total no. of specimen</th>
<th>No. (%) of specimens positive by BacT/Alert 3D system</th>
<th>P Value for BacT/Alert 3D system</th>
<th>No. (%) of specimens positive by L-J medium</th>
<th>P Value for L-J medium</th>
<th>No. (%) of specimens positive by Gottschalk medium</th>
<th>P Value for Gottschalk medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smear positivity score</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doubtful</td>
<td>5</td>
<td>3 (60.0)</td>
<td>&lt;0.001</td>
<td>4 (80.0)</td>
<td>&lt;0.001</td>
<td>1 (20.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1+</td>
<td>24</td>
<td>11 (45.8)</td>
<td>8 (33.3)</td>
<td>1 (29.2)</td>
<td>31 (70.5)</td>
<td>23 (52.3)</td>
<td></td>
</tr>
<tr>
<td>2+</td>
<td>44</td>
<td>34 (77.3)</td>
<td>31 (70.5)</td>
<td>23 (52.3)</td>
<td>44 (77.3)</td>
<td>44 (77.3)</td>
<td></td>
</tr>
<tr>
<td>3+</td>
<td>89</td>
<td>73 (82.0)</td>
<td>74 (83.1)</td>
<td>68 (76.4)</td>
<td>133 (82.2)</td>
<td>133 (82.2)</td>
<td></td>
</tr>
<tr>
<td>4+</td>
<td>157</td>
<td>130 (82.8)</td>
<td>132 (84.1)</td>
<td>133 (82.2)</td>
<td>133 (82.2)</td>
<td>133 (82.2)</td>
<td></td>
</tr>
<tr>
<td>Storage time (days)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16–30</td>
<td>8</td>
<td>6 (75.0)</td>
<td>0.050</td>
<td>7 (87.5)</td>
<td>0.07</td>
<td>7 (87.5)</td>
<td>0.111</td>
</tr>
<tr>
<td>31–60</td>
<td>19</td>
<td>13 (63.2)</td>
<td>15 (78.9)</td>
<td>15 (78.9)</td>
<td>15 (78.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>61–90</td>
<td>47</td>
<td>41 (87.2)</td>
<td>41 (87.2)</td>
<td>41 (87.2)</td>
<td>41 (87.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>91–120</td>
<td>69</td>
<td>53 (76.8)</td>
<td>58 (84.1)</td>
<td>58 (84.1)</td>
<td>58 (84.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>121–150</td>
<td>65</td>
<td>49 (75.4)</td>
<td>51 (79.5)</td>
<td>41 (66.2)</td>
<td>41 (66.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>151–180</td>
<td>111</td>
<td>90 (81.1)</td>
<td>77 (69.4)</td>
<td>77 (69.4)</td>
<td>77 (69.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sputum vol (ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2</td>
<td>97</td>
<td>78 (80.4)</td>
<td>0.019</td>
<td>68 (70.1)</td>
<td>0.023</td>
<td>55 (56.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2–5</td>
<td>222</td>
<td>173 (77.9)</td>
<td>181 (81.5)</td>
<td>181 (81.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* n = 319.
TABLE 3. Effects of smear positivity after decontamination, storage time of sputum, and sputum volume on time to detection of *M. tuberculosis* growth

<table>
<thead>
<tr>
<th>Predictor</th>
<th>BacT/Alert 3D system</th>
<th>L-J medium</th>
<th>Gottsacker medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. (%) of positive samples</td>
<td>Median TTD (days) (range)</td>
<td>P (trend)</td>
</tr>
<tr>
<td><strong>Smear positivity score</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doubtful</td>
<td>3 (1.2)</td>
<td>26 (21-40)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1+</td>
<td>11 (4.4)</td>
<td>23 (15-48)</td>
<td></td>
</tr>
<tr>
<td>2+</td>
<td>34 (13.5)</td>
<td>18 (6-28)</td>
<td></td>
</tr>
<tr>
<td>3+</td>
<td>73 (29.1)</td>
<td>16 (6-46)</td>
<td></td>
</tr>
<tr>
<td>4+</td>
<td>138 (51.8)</td>
<td>10 (6-30)</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>251 (100.0)</td>
<td>14 (6-48)</td>
<td></td>
</tr>
<tr>
<td><strong>Storage time (days)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16-20</td>
<td>6 (2.4)</td>
<td>11 (6-15)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>21-60</td>
<td>12 (4.8)</td>
<td>8 (6-15)</td>
<td></td>
</tr>
<tr>
<td>61-90</td>
<td>41 (16.3)</td>
<td>10 (6-48)</td>
<td></td>
</tr>
<tr>
<td>91-120</td>
<td>53 (21.1)</td>
<td>11 (6-23)</td>
<td></td>
</tr>
<tr>
<td>121-150</td>
<td>40 (14.9)</td>
<td>15 (6-42)</td>
<td></td>
</tr>
<tr>
<td>151-180</td>
<td>90 (35.8)</td>
<td>8 (8-46)</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>251 (100.0)</td>
<td>14 (6-48)</td>
<td></td>
</tr>
<tr>
<td><strong>Sputum vol (ml)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>8 (3.2)</td>
<td>26 (17-46)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1</td>
<td>38 (15.1)</td>
<td>22 (6-36)</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>32 (12.8)</td>
<td>21 (13-40)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>35 (13.9)</td>
<td>12 (7-48)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>25 (10.0)</td>
<td>13 (7-30)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>14 (5.6)</td>
<td>13 (6-42)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>99 (39.4)</td>
<td>10 (6-35)</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>251 (100.0)</td>
<td>14 (6-48)</td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.001 for the BacT/Alert system versus L-J medium and *P < 0.001 for the BacT/Alert system versus Gottsacker medium (by Wilcoxon matched-pairs test).

287/319 specimens (90.0%). The World Health Organization recommends that when specimens are likely to be exposed to room temperature for more than 48 h, an equal volume of either 0.6% CPB or 1% CPC should be added to homogenize and decontaminate the sample (17, 18). However, once CPB or CPC has been added to a specimen, refrigeration as a storage method is no longer possible; as these preservatives are likely to crystallize and become inactive (9). Once preserved specimens have reached the culture laboratory, a centrifugation step without refrigeration is necessary to remove the preservative prior to culture (12).

In addition, previous studies have shown the negative effects of CPC on microscopic examination and culture systems, including (i) a significant reduction in the detection of AFB with Ziehl-Neelsen staining (14, 16); (ii) an inhibition of mycobacterial growth, especially when inoculated into culture media, including Middlebrook 7H9 and 7H110 media, which have an insufficient neutralizing activity for this quaternary ammonium compound (17); and (iii) a negative effect on the BacTec MGIT 960 system, decreasing the mycobacterial detection rate and increasing the time required for mycobacterial growth readings (13). Therefore, these findings suggest a need for a simpler and cost-effective alternative to the current WHO recommendations for conducting epidemiological and drug resistance studies in resource-poor settings.

According to World Health Organization protocols for the surveillance of drug resistance, two sputum specimens should be collected to ensure a high yield of positive cultures (19). Epidemiological and drug resistance studies are expensive and technically demanding, due in part to this requirement. This recommendation has a considerable impact, as it necessitates additional logistics of storage, transportation, and dual-laboratory processing for culturing. Our results demonstrate an acceptably high yield of positive cultures from a single morning sputum specimen, with considerable time and expense savings.

Our study revealed that increasing the storage time at 20°C without the addition of preservatives had no significant effect on the rate of recovery of *M. tuberculosis*. However, in other studies, an increase in the storage time of up to 7 days at room temperature resulted in a reduced rate of recovery of *M. tuberculosis* and rising contamination rates (9). In this study, a higher degree of smear positivity was highly predictive of a positive culture result (*P < 0.001*) by all culture methods. This result was in agreement with the findings of another study, where specimen smear positivity was a factor in the recovery of *M. tuberculosis* (10). The volume of the sputum used for decontamination and homogenization was significantly predictive of a positive culture with L-J medium (*P = 0.023*) and Gottsacker medium (*P < 0.001*) but not by the BacT/Alert 3D system. This difference in the volume of the specimen and the predictive value of a positive culture among solid media and the BacT/Alert 3D system might be due to differences in the
amounts of decontaminated specimen inoculated into the media. The amount of the specimen inoculated into the BacT/Alert 3D system is 2.5 times higher than the amount inoculated into solid media. Moreover, in the BacT/Alert 3D system, small-volume specimens had a higher degree of culture positivity than specimens with a larger volume (Table 2). This might be due to the lower contamination rate, 297 (2.1%) of samples, with a smaller volume (<2 ml) than the contamination rate, 40/222 (18.0%) of samples, with a larger volume (2 to 5 ml). The higher contamination rate for the larger volume might be due to the higher load of contaminants in the larger sputum volume that could overcome the effect of the decontamination solution and antibiotic supplements in the fluid culture system.

In agreement with data from other studies (5, 6), the BacT/Alert 3D system showed a significant advantage over 1-3 and Gottsacker media in the early detection of M. tuberculosis (both P < 0.001) because of its built-in colorimetric sensor of early bacterial growth in the culture broth. However, the BacT/Alert 3D system has shown a higher contamination rate than those of Lowenstein-Jensen and Gottsacker media. This is in agreement with the culture results of the spuIn processed for routine patient diagnosis in this mycobacteriology laboratory and the results reported previously in another study (11). The higher degree of contamination of the BacT/Alert system has a considerable effect on the time to identification and cost, as it necessitates additional laboratory processing for culturing. However, these effects were minimized by the use of the BacT/Alert 3D system together with Lowenstein-Jensen and Gottsacker solid media.

In conclusion, single morning sputum specimens collected from smear-positive patients at remote settings, frozen at −20°C for long periods of time without the addition of chemical preservatives, can yield a high level of positive culture results. These findings suggest an alternative method of sputum storage with potential logistic simplification and cost savings for epidemiological and drug resistance studies in low-resource countries. Specimens were not kept at −20°C while in transport, which might support the growth of contaminants and decrease the viability of M. tuberculosis. Therefore, sample transport would require the addition of dry ice instead of ice packs to keep the specimens at a lower temperature during delivery. The level of smear positivity of patients included in this study was high; therefore, further studies are required to confirm these findings with other patients with low-level smear positivity and smear-negative pulmonary tuberculosis. Additionally, a sufficient volume of sputum specimens should be collected from smear-positive TB patients and stored at −20°C prior to transport to the mycobacteriology laboratory for culturing.

ACKNOWLEDGMENTS

This study was carried out with financial support obtained from the Fraunhofer Institute for Cell Therapy and Immunology, Leipzig, Germany; the German Academic Exchange Service (DAAD); and the University of Gondar, Gondar, Ethiopia.

We acknowledge the data collectors and the study participants from all study areas in Northwest Ethiopia. We also give our appreciation to Elisabeth Kuzecwey for her kindly assistance during sputum processing, culturing, and species identification.

REFERENCES

First- and second-line anti-tuberculosis drug resistance in Northwest Ethiopia

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**SUMMARY**

**SETTING:** Gondar Hospital, Gondar Health Centre, Metemma Hospital, Bahir Dar Hospital and Debere Markos Hospital in Northwest Ethiopia.

**OBJECTIVE:** To assess the level of and risk factors for first- and second-line drug resistance among tuberculosis (TB) patients.

**DESIGN:** Drug susceptibility testing (DST) against first-line drugs, including isoniazid (INH), rifampicin (RMP), streptomycin (SM), ethambutol (EMB) and pyrazinamide (PZA), was performed using the Bact/ALERT 3D system. DST against second-line drugs, including fluoroquinolones and aminoglycosides/cyclopeptides, was performed using GenoType MTBDRsl.

**RESULTS:** Of 260 Mycobacterium tuberculosis isolates, 41 (15.8%) were resistant to at least one first-line drug, 13 (5.0%) were multidrug-resistant (MDR) and 9 (3.5%) were resistant to all first-line drugs. Any resistance to INH, RMP, SM, EMB and PZA was respectively 36 (13.8%), 15 (5.8%), 26 (10.0%), 19 (7.3%) and 12 (4.6%). Of 214 new and 46 previously treated cases, respectively 8 (3.7%) and 5 (10.9%) were MDR. All isolates were susceptible to all second-line drugs.

**CONCLUSION:** A substantial number of new and previously treated cases harbour MDR-TB. We recommend DST at least for previously treated cases, patients who remain smear-positive at the end of the second month of treatment and patients in close contact with MDR-TB cases. Improved infection control measures need to be implemented in Ethiopia.

**KEY WORDS:** tuberculosis; drug resistance; first-line anti-tuberculosis drugs; second-line anti-tuberculosis drugs

TUBERCULOSIS (TB) is a serious global public health problem and a major cause of death due to infectious disease. High TB prevalence is assumed to result from poverty, the human immunodeficiency virus epidemic, and inadequate diagnosis and treatment. Ethiopia ranks seventh among the world’s 22 high TB burden countries. The country had 314 267 TB cases in 2007, with an estimated incidence of 378 cases per 100 000 population. Ethiopia has a total population of about 74 million, the second largest proportion of the country’s population is found in the Amhara Region (23.3%), after the Oromia Region (36.7%).

According to the World Health Organization, Mycobacterium tuberculosis resistance to one or more agents commonly used to treat TB, including isoniazid (INH), rifampicin (RMP), streptomycin (SM), ethambutol (EMB) or pyrazinamide (PZA), is of great concern. The 2009 WHO report showed that the overall proportion of multidrug-resistant tuberculosis (MDR-TB), defined as resistance to at least INH and RMP, was 5.3%, ranging from 0% to 35% of reported TB cases. The number of countries reporting at least one case of extensively drug-resistant TB (XDR-TB) had risen to 6 by January 2010, although this change is probably a result of increased efforts to identify XDR-TB rather than an increase in prevalence or distribution of the disease.

In Ethiopia, a countrywide anti-tuberculosis drug resistance survey conducted in 2005 showed a prevalence of MDR-TB of respectively 1.6% and 11.8% among new and previously treated TB cases. According to the 2008 WHO report, 5825 MDR-TB cases (4964 among newly diagnosed and 861 among previously treated TB cases) were estimated to have occurred in 2006 in Ethiopia. The Ethiopian Ministry of Health therefore started a treatment programme for MDR-TB patients in February 2009 at St Peter Hospital, in the capital Addis Ababa, and in April 2010 at Gondar University Hospital, Northwest Ethiopia.

In Ethiopia, the treatment regimens for Category I and Category II (retreatment regimen) TB cases are respectively 2(RHEZ)/4(RH) and 2(SRHEZ)/
The standard treatment regimen for MDR-TB consists of 6 months of EMB, PZA, KM (AMK), IVX, ETH and CS followed by 12 months of EMB, PZA, IVX, ETH and CS. All patients with confirmed MDR-TB are hospitalised for the first 4-8 weeks. The main criteria for discharge include sputum smear conversion, the patient’s general condition and a satisfactory follow-up plan. To design appropriate standardised regimens and increase the success of control programmes, it is important to understand the drug resistance patterns in the country. However, culture and drug susceptibility testing (DST) for mycobacteria are performed only in the National Reference Laboratory in the capital, Addis Ababa, and only limited data on drug-resistant TB are available in Ethiopia. There is therefore a need to study the extent of drug-resistant TB in Ethiopia to advocate and develop effective control strategies.

The present study aimed to assess the level of and risk factors for resistance to first- and second-line antituberculosis drugs among new and previously treated smear-positive TB patients in the Amhara Region of Northwest Ethiopia.

MATERIALS AND METHODS

Study design, area and study period

A cross-sectional study was conducted between March 2009 and July 2009 among smear-positive pulmonary TB patients diagnosed at the Gondar Hospital, the Gondar Health Centre, the Metemma Hospital, the Bahir Dar Hospital and the Debre Markos Hospital. Diagnosis of smear-positive TB was based on national guidelines for microscopic examination of TB. Direct smears were prepared from three sputum specimens and stained using Ziehl-Neelsen for microscopic examination. Once diagnosed, informed consent was obtained from study subjects and a single morning sputum sample and 5 ml venous blood sample were collected prior to commencing antituberculosis treatment. A careful examination of patient history for previous anti-tuberculosis treatment using a structured questionnaire was used to classify patients as ‘new’ and ‘previously treated’ TB cases.

Institutional ethical clearance was obtained from the research and publication committee of Gondar University, Addis Ababa, Ethiopia.

Storage and transport of sputum specimens

Specimens were kept at −20°C in the TB laboratory of each hospital until transportation to the mycobacteriology laboratory at the Institute of Medical Microbiology and Epidemiology of Infectious Diseases, University Hospital of Leipzig, Leipzig, Germany.

Isolation and identification of mycobacteria

Specimens were processed and cultured according to the Deutsches Institut für Normung (DIN) recommendations using Löwenstein-Jensen (LJ) media, Gottsacker media (Artelt-ENCLIT GmbH, Wyhra, Germany) and Bact/ALERT 3D system (BioMérieux, Marcy l’Etoile, France). Isolates were identified by DNA hybridisation technology (GenoType® MTB-C; Hain Lifescience, Nehren, Germany) following the manufacturer’s instructions.

First-line drug susceptibility testing

DST against first-line drugs, including INH, RMP, SM, EMB and PZA, was performed with the Bact/ALERT 3D system according to the methods developed for the MB/BacT system. The final drug concentrations in the test bottles were 1 μg/ml for INH, RMP and SM, and 2 μg/ml for EMB. Unlike the previous methods developed for the MB/BacT system, the final drug concentration of PZA was 200 μg/ml, as the pH of the medium in the bottle was adjusted to 6.0 by the addition of 1 ml of 0.75 m monopotassium phosphate. Furthermore, the mycobacteria process bottles were loaded into the system as mycobacteria blood bottles to inactivate the so-called delta-algorithm of the system for growth detection and thus prevent false drug-resistant results. An organism was determined to be resistant to a drug when the drug-containing bottle had a time to detection (TDT) that was less than or equal to the TDT of the 1% control.

Second-line drug susceptibility testing

DST against second-line drugs, including fluoroquinolones (FQs; i.e., ofloxacin [OFX] and moxifloxacin [MX]) and aminoglycosides (AM)/cyclopeptides (CM); capreomycin [CPR], viomycin/KM and AMK), was performed using DNA hybridisation technology on nitrocellulose strips (GenoType® MTBDRsl) according to the manufacturer’s instructions.

Human immunodeficiency virus testing

Patients’ serum samples were screened for human immunodeficiency virus (HIV) 1 and HIV-2 using the Vironostika HIV Uni-Form II Ag/Ab enzyme-linked immunosorbent assay (ELISA) kit (bioMérieux, Boxtel, The Netherlands) following the manufacturer’s instructions.

Definitions

The following WHO-recommended definitions were used for resistant cases:

Resistance among new cases: the presence of resistant isolates of M. tuberculosis in patients who had never been treated with antituberculosis drugs or who had received treatment for less than 1 month.

Resistance among previously treated cases: the presence of resistant isolates of M. tuberculosis in
patients who had previously been treated for at least 1 month.
MDR-TB: TB with strains resistant to at least INH and RMP.
XDR-TB: MDR-TB with additional resistance to an FQ and a second-line injectable agent (i.e., AMK, KM or CPM).

Statistical analysis
All laboratory data were entered, cleared and analysed using SPSS version 13 (SPSS Inc, Chicago, IL, USA). The standard χ² tests and multivariate analysis using a logistic regression model were used to assess statistical relationships between predisposing factors and drug-resistant TB. P < 0.05 was considered statistically significant.

RESULTS
A total of 260 M. tuberculosis isolates from smear-positive pulmonary TB patients were included for DST in this study. The median age of the study subjects was 28.0 years (range 7–75), and 67.3% were in the 16–35 year age group. Of all 260 study subjects, 17.7% were previously treated cases, 25.4% were HIV co-infected, 58.8% were male, 54.2% were urban residents, 41.2% were illiterate and 25.4% were farmers (Table 1).

Patterns of resistance to first-line anti-tuberculosis drugs
Of the 260 M. tuberculosis isolates, 41 (15.8%) strains presented resistance to at least one drug, 13 (5.0%) were MDR strains and 9 (3.5%) were resistant to all first-line drugs. Any resistance to INH, RMP, SM, EMB and PZA was respectively 36 (13.8%), 15 (5.8%), 26 (10.0%), 19 (7.3%) and 12 (4.6%). Monoresistance to INH, RMP and SM was respectively 9 (3.5%), 2 (0.8%) and 4 (1.5%). However, monoresistance to EMB and PZA was not observed in any strains (Tables 2 and 3).

Resistance among new cases
Of the 214 new cases, resistant strains were isolated in 23 (10.7%) patients. MDR-TB was detected in 8 (3.7%) strains, and 6 (2.8%) were resistant to all first-line drugs. Monoresistance to INH and SM was observed in respectively 6 (2.8%) and 3 (1.4%) cases. Monoresistance to RMP, EMB and PZA was not observed among new cases.

Resistance among previously treated cases
Of the 46 previously treated TB cases, resistant strains were isolated in 18 (39.1%) patients. MDR-TB was detected in 5 (10.9%) strains and 3 (6.5%)

Table 1 Characteristics of the study subjects in Northwest Ethiopia (n = 260)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Previous anti-tuberculosis treatment</td>
<td>214 (82.3)</td>
</tr>
<tr>
<td>Yes</td>
<td>46 (17.7)</td>
</tr>
<tr>
<td>HIV status</td>
<td>194 (74.6)</td>
</tr>
<tr>
<td>Negative</td>
<td>66 (25.4)</td>
</tr>
<tr>
<td>Positive</td>
<td>153 (58.8)</td>
</tr>
<tr>
<td>Female</td>
<td>107 (41.2)</td>
</tr>
<tr>
<td>Age group, years</td>
<td>7 (2.7)</td>
</tr>
<tr>
<td>&lt;15</td>
<td>95 (36.5)</td>
</tr>
<tr>
<td>16–25</td>
<td>80 (30.5)</td>
</tr>
<tr>
<td>26–35</td>
<td>43 (16.5)</td>
</tr>
<tr>
<td>36–45</td>
<td>35 (13.5)</td>
</tr>
<tr>
<td>Place of residence</td>
<td>119 (45.8)</td>
</tr>
<tr>
<td>Rural</td>
<td>141 (54.2)</td>
</tr>
<tr>
<td>Urban</td>
<td>107 (41.2)</td>
</tr>
<tr>
<td>Educational status</td>
<td>153 (58.8)</td>
</tr>
<tr>
<td>Illiterate</td>
<td>66 (25.4)</td>
</tr>
<tr>
<td>Literate</td>
<td>42 (16.2)</td>
</tr>
<tr>
<td>Employment status</td>
<td>23 (8.8)</td>
</tr>
<tr>
<td>Student</td>
<td>3 (1.4)</td>
</tr>
<tr>
<td>Farmer</td>
<td>3 (1.4)</td>
</tr>
<tr>
<td>Housewife</td>
<td>3 (1.4)</td>
</tr>
<tr>
<td>Government employee</td>
<td>3 (1.4)</td>
</tr>
<tr>
<td>Merchant</td>
<td>3 (1.4)</td>
</tr>
<tr>
<td>Daily labour</td>
<td>3 (1.4)</td>
</tr>
<tr>
<td>Other</td>
<td>3 (1.4)</td>
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<tr>
<td>Health institute</td>
<td>3 (1.4)</td>
</tr>
<tr>
<td>Gondar Hospital</td>
<td>113 (43.5)</td>
</tr>
<tr>
<td>Gondar Health Centre</td>
<td>37 (14.2)</td>
</tr>
<tr>
<td>Metema Hospital</td>
<td>9 (3.5)</td>
</tr>
<tr>
<td>Bahir Dar Hospital</td>
<td>28 (17.9)</td>
</tr>
<tr>
<td>Debark Hospital</td>
<td>30 (11.5)</td>
</tr>
</tbody>
</table>

HIV = human immunodeficiency virus.

Table 2 Pattern of resistance to first-line anti-tuberculosis drugs in new and previously treated tuberculosis cases

<table>
<thead>
<tr>
<th>Drug resistance</th>
<th>New cases (n = 214)</th>
<th>Previously treated cases (n = 46)</th>
<th>Total (N = 260)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any resistance</td>
<td>21 (9.6)</td>
<td>15 (32.6)</td>
<td>36 (13.8)</td>
</tr>
<tr>
<td>INH</td>
<td>8 (3.7)</td>
<td>7 (15.2)</td>
<td>15 (5.8)</td>
</tr>
<tr>
<td>RMP</td>
<td>14 (6.5)</td>
<td>12 (26.1)</td>
<td>26 (10.0)</td>
</tr>
<tr>
<td>SM</td>
<td>6 (2.8)</td>
<td>3 (6.5)</td>
<td>9 (3.5)</td>
</tr>
<tr>
<td>EMB</td>
<td>6 (2.8)</td>
<td>3 (6.5)</td>
<td>9 (3.5)</td>
</tr>
<tr>
<td>PZA</td>
<td>6 (2.8)</td>
<td>3 (6.5)</td>
<td>9 (3.5)</td>
</tr>
<tr>
<td>Monoresistance</td>
<td>2 (0.9)</td>
<td>9 (20.5)</td>
<td>11 (4.2)</td>
</tr>
<tr>
<td>INH</td>
<td>2 (0.9)</td>
<td>9 (20.5)</td>
<td>11 (4.2)</td>
</tr>
<tr>
<td>RMP</td>
<td>2 (0.9)</td>
<td>9 (20.5)</td>
<td>11 (4.2)</td>
</tr>
<tr>
<td>SM</td>
<td>2 (0.9)</td>
<td>9 (20.5)</td>
<td>11 (4.2)</td>
</tr>
<tr>
<td>EMB</td>
<td>2 (0.9)</td>
<td>9 (20.5)</td>
<td>11 (4.2)</td>
</tr>
<tr>
<td>PZA</td>
<td>2 (0.9)</td>
<td>9 (20.5)</td>
<td>11 (4.2)</td>
</tr>
</tbody>
</table>

Resistance to

Only two drugs

INH + RMP | 2 (0.9) | 9 (20.5) | 11 (4.2) |
INH + SM | 2 (0.9) | 9 (20.5) | 11 (4.2) |
INH + EMB | 2 (0.9) | 9 (20.5) | 11 (4.2) |
INH + RMP + SM | 2 (0.9) | 9 (20.5) | 11 (4.2) |
INH + RMP + EMB | 2 (0.9) | 9 (20.5) | 11 (4.2) |
INH + RMP + SM + EMB | 2 (0.9) | 9 (20.5) | 11 (4.2) |
INH + RMP + SM + EMB + PZA | 2 (0.9) | 9 (20.5) | 11 (4.2) |
INH + RMP + SM + EMB + PZA | 2 (0.9) | 9 (20.5) | 11 (4.2) |
INH + RMP + SM + EMB + PZA | 2 (0.9) | 9 (20.5) | 11 (4.2) |
INH + RMP + SM + EMB + PZA | 2 (0.9) | 9 (20.5) | 11 (4.2) |
INH + RMP + SM + EMB + PZA | 2 (0.9) | 9 (20.5) | 11 (4.2) |
INH + RMP + SM + EMB + PZA | 2 (0.9) | 9 (20.5) | 11 (4.2) |
INH + RMP + SM + EMB + PZA | 2 (0.9) | 9 (20.5) | 11 (4.2) |
were resistant to all first-line drugs. Monoresistance to INH, RMP and SM was observed in respectively 3 (6.5%), 2 (4.3%) and 1 (2.2%) cases. Monoresistance to EMB and PZA was not observed among previously treated cases.

Second-line drug resistance
All the 260 isolates, including the 13 MDR-TB strains, were susceptible to all second-line anti-tuberculosis drugs tested, including FQs (OFX and MFX) and AM/CM (CPM, viomycin/KM and AMK).

Risk factors associated with drug resistance
As shown in Tables 3 and 4 and the Figure, history of previous anti-tuberculosis treatment was identified as a significant risk factor for resistance to one or more anti-tuberculosis drugs (odds ratio [OR] 5.1, P < 0.001) and MDR-TB (OR 3.1, P = 0.044). Patients diagnosed at the Metemma Hospital showed a significantly higher level of resistance to one or more anti-tuberculosis drugs compared with those from the Debre Markos Hospital (OR 14.5, P = 0.031). The HIV status of new and previously treated cases was not significantly associated with resistance. The Figure shows that history of previous anti-tuberculosis treatment was identified as a significant risk factor for any resistance to INH (P < 0.001), RMP (P = 0.002), SM (P < 0.001) and EMB (P = 0.023). However, it does not appear to be a significant risk factor for PZA resistance.

DISCUSSION
This study was mainly aimed at determining the level and risk factors for first- and second-line drug resistance among new and previously treated TB cases in Northwest Ethiopia. The overall rate of resistance to first-line drugs among new cases, 10.7%, is comparable to the rates reported in Ivory Coast (13.4%) and in the Central African Republic (16.4%). However, higher levels of resistance were reported in Ethiopia (26.9%) and 25% and Cameroon (31.8%) and
Ghana (54.4%). The overall rate of resistance to first-line drugs among previously treated TB cases was 39.1%. Other studies have reported rates of 48.7% and 85.7% in Ethiopia, 17 56% in Nigeria, 18 58.2% in Yaounde, Cameroon, 19 and 63% in the Central African Republic. 20 The higher level of drug resistance reported in Addis Ababa compared to the present study might be due to the difference in study subjects, as the previous study included only TB patients referred to the TB specialized hospital in Ethiopia for treatment and DST due to suspicion of MDR-TB.

In the present study, the overall rates of MDR-TB in new and previously treated cases were respectively 3.7% and 10.9%. The countrywide anti-tuberculosis drug resistance survey in 2005 showed a lower rate of MDR-TB, of 1.6%, among new cases and a similar rate, 11.8%, among previously treated TB cases. A report from Addis Ababa showed a lower rate of MDR-TB, 2.3% (only one patient), among new cases and a higher rate, 71.4%, among previously treated patients. Other studies in Ethiopia also showed lower levels of MDR-TB among new cases, 1.2%, 20 0.6% 21 and 0%. 22 Studies in other African countries reported rates of MDR-TB of 0.5% to 3.5% in new cases, and of 1.4% to 18.2% in previously treated cases. 23-26 A higher level of MDR-TB among new cases in this study than in previous studies in Ethiopia may reflect the wider spread of MDR-TB and the fact that the control measures in place to prevent the development and the transmission of drug-resistant TB are insufficient in study areas. These findings may also indicate a critical situation for drug-resistant TB control measures in the country. The DOTS strategy has been implemented in almost all districts in Ethiopia, and is being scaled up to nearly 100% of the

Table 4: Risk factors for MDR-TB in Northwest Ethiopia (N = 263)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>MDR-TB (%)</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>13 (5.0)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Previous TB treatment</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>No*</td>
<td>8 (3.7)</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>Yes</td>
<td>5 (19.6)</td>
<td>3.1 (1.0-10.1)</td>
<td>0.044</td>
</tr>
<tr>
<td>HIV status</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Negative*</td>
<td>9 (4.6)</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>Positive</td>
<td>4 (6.1)</td>
<td>1.3 (0.4-4.5)</td>
<td>0.648</td>
</tr>
<tr>
<td>Sex</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Male*</td>
<td>7 (4.6)</td>
<td>1.2 (0.4-3.8)</td>
<td>0.707</td>
</tr>
<tr>
<td>Female</td>
<td>6 (5.6)</td>
<td>—</td>
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<td>Age group, years &lt;15*</td>
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<tr>
<td>16-25</td>
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<td>2.7 (0.3-22.8)</td>
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<tr>
<td>26-35</td>
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<td>0.9 (0.1-9.9)</td>
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<tr>
<td>36-45</td>
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<td>2.6 (0.3-25.7)</td>
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<tr>
<td>Place of residence</td>
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<tr>
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<td>4 (3.4)</td>
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<tr>
<td>Urban</td>
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<td>0.2 (0.1-1.1)</td>
<td>0.072</td>
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<tr>
<td>Literate*</td>
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<td>Employment status</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Student*</td>
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<td>1.0</td>
<td>—</td>
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<tr>
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<td>1.3 (0.1-14.2)</td>
<td>0.857</td>
</tr>
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<td>Housewife</td>
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<td>1.0 (0.1-16.1)</td>
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<td>Government employee</td>
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<tr>
<td>Daily labour</td>
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<td>4.2 (0.5-39.4)</td>
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<tr>
<td>Others</td>
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<td>3.3 (0.2-57.4)</td>
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<td>—</td>
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<tr>
<td>Gonder Hospital*</td>
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<td>1.0</td>
<td>—</td>
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<tr>
<td>Gonder Health Centre</td>
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<td>1.3 (0.3-5.5)</td>
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<tr>
<td>Metemma Hospital</td>
<td>1 (11.1)</td>
<td>1.9 (0.2-17.3)</td>
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<tr>
<td>Bahir Dar Hospital</td>
<td>2 (2.8)</td>
<td>0.4 (0.1-2.2)</td>
<td>0.313</td>
</tr>
</tbody>
</table>

*Reference category. MDR-TB = multidrug-resistant tuberculosis; OR = odds ratio; CI = confidence interval; HIV = human immunodeficiency virus.

Figure: Comparison of drug resistance among new and previously treated cases. INH = isoniazid; RMP = rifampicin; SM = streptomycin; EMB = ethambutol; PZA = pyrazinamide; MDR-TB = multidrug-resistant tuberculosis.
143 hospitals and 699 health centres providing TB diagnostic and treatment services. However, the DOTS strategy case detection rate remains low, at 28% in 2007, compared with the WHO target of 70% detection. The limited diagnostic capacity in Ethiopia remains a challenge for improving case detection rates. Drugs are provided only through the national procurement and distribution system to the public and private sectors, and the rapidly expanding private health sector is being involved in TB control activities. Infection control measures in Ethiopia remain poor and need to be improved.

Patients with a history of previous treatment at the time of diagnosis had respectively five patients with three times greater odds of presenting resistance to one or more first-line drugs and multidrug resistance (Tables 3 and 4). Similar results have also been reported by other studies. The higher level of drug resistance among previously treated TB cases than in new TB cases might be due to inappropriate or irregular drug supply or to patient non-adherence to treatment regimes, thereby favouring the selection of spontaneously mutated M. tuberculosis strains. Patients diagnosed at the Metemma Hospital had 14 times greater odds of having resistance to one or more first-line drugs than patients from the Debre Markos Hospital (Table 3). This might be due to differences in implementation of infection control measures in different parts of the country.

Among new cases, the highest rate of monoresistance observed in the study was associated with INH (2.8%). The highest level of INH monoresistance among new cases reported in other studies was 2%1 and 2.3%15 in Ethiopia, and 7.9%26 in Mozambique. Monoresistance to SM was only 1.4% among new cases in this study, lower than rates reported in other studies: 10.2%,33 11.4%11 and 17.4%14 in Ethiopia, and 2.5%,24 5.9%24 and 7.0%23 in other African countries. No monoresistance to RMP, EMB and PZA among new cases was detected in this study. Other studies in Ethiopia also reported an absence of monoresistance to RMP and EMB,15 or low levels of monoresistance to RMP (1.0%) and EMB (0.1%).1 In this study, monoresistance to EMB and PZA was not observed in any strain. To the best of our knowledge, this study is the first report in Ethiopia to assess the level of PZA resistance in Ethiopia.

The high burden of MDR-TB in Ethiopia messages that effective second-line drug treatment regimes. Studies on the levels of first- and second-line drug resistance are useful in devising effective treatment regimes, as they permit the selection of drugs with a low resistance rate to obtain good cure rates and avoid amplification of resistance. In the present study, the results of second-line DST were encouraging in that all isolates were susceptible to all second-line drugs tested. This is in agreement with other reports that showed absence of resistance to second-line drugs in Zambia and low levels of second-line drug resistance and absence of XDR-TB strains among MDR M. tuberculosis isolates in Rwanda. In another study in Addis Ababa, XDR-TB was detected in two isolates (4.4%). The absence of resistance to second-line drugs in our study may be indicative of the low use of and access to these drugs in Northwest Ethiopia.

The HIV/AIDS (acquired immune-deficiency syndrome) situation in the Amhara Region is one of the worst in the country. HIV/AIDS prevalence was estimated at 2.8% in 2009 and at 2.9% in 2010 in the region, which is higher than the prevalence nationwide, estimated at 2.3% in 2009 and 2.4% in 2010. In the present study, the prevalence of HIV co-infection in smear-positive TB patients was 25.4%. In a previous study, a higher rate of TB-HIV co-infection, of 52.1% was reported from Gondar Hospital, Ethiopia. However, the previous study was conducted in both pulmonary TB (smear-positive and smear-negative) and extra-pulmonary TB patients. Excluding smear-negative and extra-pulmonary TB patients in the present study probably excluded several cases of TB patients co-infected with HIV. In agreement with other reports from sub-Saharan Africa and the Global Project on Drug Resistance, HIV infection status in new and previously treated cases in this study was not significantly associated with resistance to anti-tuberculosis drugs. The high HIV prevalence in the community does not appear to be a significant factor selectively driving drug-resistant TB development and transmission in this study. Rather, high HIV prevalence is increasing the susceptibility of the population to both drug-susceptible and drug-resistant TB.

CONCLUSIONS

A substantial number of new and previously treated cases in Northwest Ethiopia harbour MDR-TB. XDR-TB was not detected in this study. Previously treated cases and patients diagnosed at Metemma Hospital showed a significantly higher level of resistance to one or more anti-tuberculosis drugs. TB laboratories need to be rebuilt and equipped to conduct culture and DST for at least previously treated cases of TB, patients who remain smear-positive at the end of the second month of WHO Category 1 treatment, and new patients in close contact with MDR-TB cases. As the low case detection rate results in long periods of contact between infectious individuals and the community, strengthening of laboratory diagnostic capacity and other intensified case-finding strategies are urgently needed to increase the case detection rate. The existing decentralised MDR-TB treatment service in Ethiopia needs to be decentralised to cope with the large numbers of patients requiring treatment and to keep them on treatment for the full time required. Improved infection control measures need to be implemented in Ethiopia to reduce the risk of TB transmission in the community.
Acknowledgements

The authors thank data collectors and study participants from all study areas in Northwest Ethiopia. They express their appreciation to E. Krawczyk for her kind assistance during drug susceptibility testing. This study was carried out with financial support from the Fraunhofer Institute for Cell Therapy and Immunology, Leipzig, Germany, Institute of Medical Microbiology and Epidemiology of Infectious Diseases, University Hospital Leipzig, Germany, German Academic Exchange Service (DAAD), and University of Gondar, Ethiopia.

References


Analysis of gene mutations associated with isoniazid, rifampicin and ethambutol resistance among *Mycobacterium tuberculosis* isolates from Ethiopia

Belay Tessema1,2,3*, Joerg Beer2, Frank Emmrich1,4, Ulrich Sack3,4 and Ame C Rodloff2

**Abstract**

**Background:** The emergence of drug resistance is one of the most important threats to tuberculosis control programs. This study was aimed to analyze the frequency of gene mutations associated with resistance to isoniazid (INH), rifampicin (RMP) and ethambutol (EMB) among *Mycobacterium tuberculosis* isolates from Northwest Ethiopia, and to assess the performance of the GenoType® MTBDRplus and GenoType® MTBDRsl assays as compared to the Bact/ALERT 3D system.

**Methods:** Two hundred sixty *Mycobacterium tuberculosis* isolates from smear positive tuberculosis patients diagnosed between March 2009 and July 2009 were included in this study. Drug susceptibility tests were performed in the Institute of Medical Microbiology and Epidemiology of Infectious Diseases, University Hospital of Leipzig, Germany.

**Results:** Of 260 isolates, mutations conferring resistance to INH, RMP, or EMB were detected in 35, 15, and 8 isolates, respectively, while multidrug resistance (MDR) was present in 13 of the isolates. Of 35 INH resistant strains, 33 had mutations in the *katG* gene at Ser315Thr 1 and two strains had mutation in the *inhA* gene at C15T. Among 15 RMP resistant isolates, 11 had rpoB gene mutation at Ser315Leu, one at His526Asp, and three strains had mutations only at the wild type probes. Of 8 EMB resistant strains, two had mutations in the *embB* gene at Met306Le, one at Met306Val, and five strains had mutations only at the wild type probes. The GenoType® MTBDRplus assay had a sensitivity of 92% and specificity of 99% for INH resistance, and 100% sensitivity and specificity to detect RMP resistance and MDR. The GenoType® MTBDRsl assay had a sensitivity of 42% and specificity of 100% for EMB resistance.

**Conclusion:** The dominance of single gene mutations associated with the resistance to INH and RMP was observed in the codon 315 of the *katG* gene and codon 531 of the *rpoB* gene, respectively. The GenoType® MTBDRplus assay is a sensitive and specific tool for diagnosis of resistance to INH, RMP and MDR. However, the GenoType® MTBDRsl assay shows limitations in detecting resistance to EMB.

**Keywords:** *Mycobacterium tuberculosis*, Drug resistance, Gene mutation

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Background

According to the World Health Organization (WHO) report, the proportion of multidrug resistant tuberculosis (MDR-TB), resistant to at least isoniazid and rifampicin among new and previously treated TB cases globally ranges from 0% to 28.3% and from 0% to 61.6%, respectively [1]. In Ethiopia, the countrywide anti-TB drug resistance survey conducted in 2005 showed that the prevalence of MDR-TB was 1.6% and 11.8% among new and previously treated TB cases, respectively [2]. Moreover, 5825 MDR-TB cases were estimated to have occurred in 2006 in Ethiopia [3]. MDR-TB treatment involves prolonged use of second-line anti-TB drugs that are less effective, less tolerated, more toxic, and more expensive than first-line anti-TB drugs [4]. In high-burden TB countries, MDR-TB is only
diagnosed after prolonged treatment with first-line TB drugs and clinical recognition that treatment has failed. Treatment of drug-resistant TB with standard first-line drugs, instead of a regimen designed according to the resistance pattern has several potential adverse consequences: patients remain on inadequate treatment longer, increasing the risk of treatment failure or death; selection of drug resistant strains and patients remain infectious, promoting transmission to close contacts [5].

In Ethiopia, the treatment regimens for category I and category II (retreatment regimen) tuberculosis cases are 2 (RMP-INH-EMB-PZA)/4(RMP-INH) and 2 STM (RMP-INH-EMB-PZA)/1(RMP-INH-EMB-PZA)/5(EMB2 (RMP-INH)), respectively [6]. The standard treatment regimen for MDR-TB is 4(EMB-PZA-KM (AMB)-LFXETO-CS)/12(EMB-PZA-LFX-ETO-CS). For proper treatment and control of tuberculosis, WHO is recommending countries to expand their capacity for culture based drug-susceptibility testing (DST) and consider new, molecular-based assays for diagnosing drug resistance [7,8]. Since M. tuberculosis usually grows slowly, the identification and drug-resistance testing usually require several weeks. The gold-standard of TB diagnosis by culture takes weeks to become positive, and even with the up-to-date automated fluid culture methods it takes an average of 14 days. Another 14 days for additional testing are required to get the information on drug susceptibility [9-11]. Molecular methods of drug resistance testing, based on the identification of mutations in genes associated with drug resistance, like Genexpert MTB/RIF assay, offers an effective tool for determining drug resistance because of their high sensitivity, specificity and speed [12].

Molecular methods that have been developed to detect drug resistance include the GenoType® MTBDRplus for detection of INH and RMP resistance and the GenoType® MTBDRsl for detection of resistance against EMB, fleroxacinolone, and aminoglycosides/cyclic peptides (Hain Lifescience, Nehren, Germany). These assays are DNA strip assays that use PCR and hybridization. Mutations in katG gene and inhA gene were related to the high-level and low-level INH resistance, respectively [13]. Nearly all RMP resistant strains contain mutation of the rpoB gene, coding RNA polymerase subunit β and mutation in the embB gene was associated with EMB resistance [14,15].

In Ethiopia, culture and drug susceptibility testing (DST) for M. tuberculosis are not performed routinely in clinical microbiology laboratories. Laboratory diagnosis of TB remains in a stage of acid-fast staining. Currently, five regional laboratories are being rebuilt and equipped to perform culture and drug susceptibility testing using methods including GenoType® MTBDRplus assay. The GenoType® MTBDRplus and GenoType® MTBDRsl assays have been studied in several laboratories of other countries, however, there is a wide variation in circulating M. tuberculosis strains worldwide [16,17], and false negative results may occur due to unique genetic mutations [18-24], affecting the performance of molecular assays for drug susceptibility testing. Therefore, analysis of gene mutations associated with resistance to anti-tuberculosis drugs and assessment of the performance of molecular methods for drug resistance testing in different settings is needed to ensure acceptable performance of the assays. So far, there was no report on the frequency of gene mutations associated with resistance to INH, RMP and EMB and the applicability of these molecular assays for M. tuberculosis isolates from Ethiopia. In this study, we analyzed the frequency of gene mutations associated with resistance to INH, RMP and EMB among M. tuberculosis isolates from Northwest Ethiopia, and assessed the performance of the GenoType® MTBDRplus for detection of resistance to INH, RMP and MDR and GenoType® MTBDRsl assay for detection of EMB resistance compared to the automated, culture-based, BacT/ALERT 3D system drug susceptibility testing.

Methods

Study design, area and study period
Two hundred sixty M. tuberculosis isolates from smear positive tuberculosis patients diagnosed between March 2009 and July 2009 at Gondar Hospital, Gondar Health Center, Metemma Hospital, Bahir Dar Hospital and Debre Markos Hospital in Northwest Ethiopia were included in this study. Diagnosis of smear-positive tuberculosis was based on the national guideline for microscopic examination of tuberculosis (6): direct smears were prepared from three sputum specimens and stained by Ziehl-Neelsen staining technique for microscopic examination. Drug susceptibility tests using GenoType® MTBDRplus, GenoType® MTBDRsl and BacT/ALERT 3D system were performed at the mycobacteriology laboratory in the Institute of Medical Microbiology and Epidemiology of Infectious Diseases, University Hospital of Leipzig, Germany. Informed consent was obtained from the study subjects. Institutional ethical clearance was obtained from the research and publication committee of Gondar University, Ethiopia. Details of sputum storage, transportation, isolation and identification of the isolates have described previously [25].

GenoType® MTBDRplus and GenoType® MTBDRsl drug susceptibility testing
GenoType® MTBDRplus assay for detection of INH and RMP resistance, and GenoType® MTBDRsl assay for detection of ethambutol resistance were performed
according to the manufacturer’s instructions (Hain Life-
science GmbH, Nehren, Germany). Briefly, DNA was
extracted from cultures by heating the bacteria in a
heating block for 20 minutes at 95°C followed by sonica-
tion in ultrasonic water bath for 15 minutes. Amplifica-
tion was performed using 2.5 µl (1 unit) Taq DNA
polymerase (ROCHE, Mannheim, Germany). For the
amplification profile the instructions of the manufac-
turer were followed. Hybridization of the single-
stranded, biotin-labeled amplicons to membrane-bound
probes on the strip followed by addition of conjugate,
and substrate to detect visible band patterns on the strip
was performed manually using a shaking water bath,
Memmert-SV1422 (Memmert GmbH & CO.KG, Schwa-
bach, Germany) at 45°C. Then strips were allowed to
dry and interpreted according to the manufacturer’s recom-
pendation.

To detect high level INH resistance, the GenoType®
MTBDRplus has incorporated one wild-type (WT-315)
and two mutation-type probes specific for mutation Ser315Thr1 and Ser315Thr2 of the katG gene. For
detection of low-level INH resistance this assay has two
wild-type probes (WT-15.16 and WT-8) and four
mutation-type probes, covering codons of C15T, A16G, T8C and T8A in the inhA gene. To detect rifampi-
cin resistance, the Genotype MTBDRplus has incorpo-
rated eight wild-type probes for the rpoB gene, covering
codons in the rpoB gene from 505 to 533, and four
other probes specific for mutations Asp516Val, His526-
Tyr, His526Asp and Ser531Leu. For detection of ethambutol resistance, the GenoType® MTBDRsl employs one
wild-type probe (WT-306) and two mutation probes specific for mutations Met306lle and Met306Val in the
embB gene.

BacT/ALERT 3D system drug susceptibility testing
Drug susceptibility testing for isoniazid, rifampicin and
ethambutol was performed by BacT/ALERT 3D system
(BioMerieux, S.A, France) according to the methods
published previously [26, 27]. The final drug concen-
tration in the test bottles was 1 µg/ml for INH and RMP,
and 2 µg/ml for EMB. Two control bottles, one with 1% control (0.5 ml of the 1:100 diluted test organisms sus-
pension) and one original control bottle without drug
were used for interpretation of the test results. M. tubercu-
losis isolate was determined to be resistant to an antibi-
otic when the drug-containing bottle had a time to
detection (TTD) that was less than or equal to the TTD
of the 1% control.

Statistical analysis
All laboratory data were entered, cleared and analyzed
using SPSS version 13 statistical package software (SPSS
Inc., Chicago, IL). The standard chi-square tests (χ2)
were used to assess statistical relationships between pre-
disposing factors and drug-resistant TB. Sensitivity, spec-
ificity, positive predictive value and negative predictive
value of the molecular methods were analyzed using
crosstabulation after arranging the results of the mole-
cular methods in the rows and gold standard, BacT/
ALERT 3D system in columns. P values of less than
0.05 were considered statistically significant.

Results
Of the 260 patients included in this study, the majority
of patients, 59% were males. The median age of the
study subjects was 28.0 years (range, 7-75 years). History
of previous treatment for tuberculosis was significantly
associated with gene mutations conferring resistance to
INH (P = 0.001), RMP (P = 0.002) and MDR (P = 0.044). HIV co-infection, gender and age of the study
subjects had no significant association with gene muta-
tions conferring resistance to INH, RMP and EMB. A
summary of patient demographic characteristics and
associated drug susceptibility pattern according to the
molecular methods is shown in Table 1.

Mutations associated with INH, RMP and EMB resistance
Mutations conferring resistance to isoniazid, rifampicin
and ethambutol were detected in 14%, 6% and 3% of
the isolates, respectively. Five percent of the isolates showed
mutation in both rpoB gene and katG gene or inhA pro-

tector region indicating that they were multidrug resis-
tant. There was no isolate that showed mutations at
both katG and inhA genes. Mutations associated with
isoniazid resistance were more often encountered as
compared to those seen in rifampicin and ethambutol.
Of 35 INH resistant strains, 94% had mutation in the
katG (codon 315) gene with amino acid change of Ser315Thr1, indicating high level resistance, while 6% of
the strains had mutation in the inhA gene, C15T, indi-
cating low level resistance. All katG gene mutations
detected at wild type probes were also present at mutant
probes, as was the case with the inhA gene mutations
(Table 2). Additionally, three strains showing resistance
to isoniazid and two strains sensitive to isoniazid by the
BacT/ALERT 3D system did not display concordant
results by GenoType® MTBDRplus even on repeat
assays (Table 3).

The rifampicin resistant isolates displayed different
mutations: 73% of the isolates had mutation at position
Ser531Leu, one isolate had mutation at His526Asp, while in three of the isolates mutation was detected only
at the wild type probes. Of the isolates with mutation
that detected only at wild type probes, one isolate had
mutation at rpoB WT2 and WT3, one isolate at rpoB
WT4 and one isolate at rpoB WT6. According to the kit
manufacturer’s recommendation, the three isolates with
Table 1 Characteristics of study subjects and their association with resistance to isoniazid, rifampicin and ethambutol based on GenoType® MTBDRplus and GenoType® MTBDRsl assays

<table>
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<tr>
<th>Characteristics</th>
<th>Number of patients</th>
<th>Anti-TB drug resistance</th>
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<tbody>
<tr>
<td></td>
<td>INH N (%)</td>
<td>P-value</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td>RMP N (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P-value</td>
</tr>
<tr>
<td>Male</td>
<td>153</td>
<td>0.526</td>
</tr>
<tr>
<td>Female</td>
<td>107</td>
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</tr>
<tr>
<td></td>
<td>6 (5.9)</td>
<td>7 (4.6)</td>
</tr>
<tr>
<td></td>
<td>6 (5.8)</td>
<td>3 (2.8)</td>
</tr>
<tr>
<td>Age (years)</td>
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<td></td>
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<td>&lt; 40</td>
<td>214</td>
<td>0.927</td>
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<td></td>
<td>11 (5.1)</td>
<td>4 (2.1)</td>
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<tr>
<td>≥ 40</td>
<td>46</td>
<td>0.348</td>
</tr>
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<td></td>
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<td>TB history</td>
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<td>New</td>
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<td></td>
<td>8 (3.7)</td>
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<td>Previously treated</td>
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<td></td>
<td>13 (28.3)</td>
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<td>Positive</td>
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<tr>
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<td>9 (4.6)</td>
<td>4 (6.1)</td>
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<td>Total</td>
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<tr>
<td></td>
<td>15 (5.8)</td>
<td>1 (1.5)</td>
</tr>
<tr>
<td></td>
<td>13 (5.0)</td>
<td>8 (3.1)</td>
</tr>
</tbody>
</table>

N number, INH isoniazid, RMP rifampicin, MDR multidrug resistance, EMB ethambutol

mutation that detected only at wild type probes were considered resistant (Table 2).

Mutations associated with ethambutol resistance were less frequent compared to those seen in isoniazid and rifampicin resistance. Of the 8 EMB resistant strains according to the molecular method, two strains had mutations in the embB (codon 306) gene with amino acid change of Met306Le and one strain had mutation in the embB gene with amino acid change of Met306Val, whereas five strains had mutation that detected only at the wild type probes (embB WT) but not at the mutant probes (Table 2). Moreover, 58% of the isolates showing resistance to ethambutol by the BacT/ALERT 3D system did not display a concordant result by GenoType® MTBDRsl assay even on repeat assays (Table 3). All isolates included in this study had no mutations conferring resistance to fluoroquinolones and aminoglycosides. This might be due to low use/access to these drugs in Northwest Ethiopia.

Performance of GenoType® MTBDRplus and GenoType® MTBDRsl assays

Compared with the automated, culture-based, BacT/ALERT 3D system drug susceptibility testing, the GenoType® MTBDRplus assay had a sensitivity of 92% and specificity of 99% for detection of INH resistance, a sensitivity of 100% and specificity of 100% for RMP resistance, and a sensitivity of 100% and specificity of 100% for multidrug resistance. The GenoType® MTBDRsl assay had a sensitivity of 42% and specificity of 100% for detection of EMB resistance (Table 3).

Table 2 Frequency of gene mutations associated with resistance to isoniazid and rifampicin by GenoType® MTBDRplus, and to ethambutol by GenoType® MTBDRsl assays

<table>
<thead>
<tr>
<th>Anti-TB-drugs</th>
<th>Number of resistant isolates</th>
<th>Patterns of gene mutations (wild-type/mutant)</th>
<th>Amino acid Changes</th>
<th>Frequency (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid</td>
<td>35</td>
<td>katGWT/katGMUT1/inhA WT1/inhA MUT1</td>
<td>Ser315Thr1</td>
<td>33</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>15</td>
<td>rpoB WT8/rpoB MUT3</td>
<td>Ser531Leu</td>
<td>11</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>8</td>
<td>embB WT/embB MUT1A</td>
<td>Met306Le</td>
<td>2</td>
</tr>
</tbody>
</table>

n number of isolates, WT wild-type, MUT mutant, ND no mutation detected at mutant probe, N/A mutant probe is not available
Table 3 Performance of GenoType® MTBDRplus assay for detection of resistance to INH, RMP and MDR and GenoType® MTBDRsl assay for detection of EMB resistance in comparison to Bact/Alert 3D system

<table>
<thead>
<tr>
<th>Molecular methods DST results</th>
<th>Bact/Alert 3D DST results</th>
<th>Sensitivity %</th>
<th>Specificity %</th>
<th>PPV %</th>
<th>NPV %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Susceptible</td>
<td>Resistant</td>
<td>Susceptible</td>
<td>Resistant</td>
<td></td>
</tr>
<tr>
<td>INH</td>
<td>222</td>
<td>3</td>
<td>91.7</td>
<td>99.1</td>
<td>94.3</td>
</tr>
<tr>
<td>RMP</td>
<td>245</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>INH + RMP (MDR)</td>
<td>247</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>EMB</td>
<td>241</td>
<td>11</td>
<td>42</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

DST drug susceptibility testing. INH isoniazid, RMP rifampicin, MDR multidrug resistance, EMB ethambutol. PPV positive predictive value. NPV negative predictive value.

Discussion

Almost all TB laboratories in Ethiopia have only been equipped with the acid-fast staining and lack resources for culture, identification and drug susceptibility testing of mycobacteria, which present a huge hindrance for tuberculosis control in the country. Therefore, there is an urgent need for laboratories to find a rapid and efficient method for TB diagnosis as a complement to the smear microscopy, and meanwhile to establish MDR-TB diagnostic route for rapid detection of drug-resistant TB. The GenoType® MTBDRplus and GenoType® MTBDRsl assays are rapid and technically simple to perform and do not require sophisticated equipment when compared with the conventional culture-based techniques. These assays have been studied in other countries. However, false negative results reported due to unique genetic mutations associated with resistance to anti-tuberculosis drugs in different countries [18-24]. Therefore, in this study, we investigated the frequency of gene mutations associated with resistance to INH, RMP and EMB and evaluated the performance of these molecular assays for detection of resistance to INH, RMP and EMB on M. tuberculosis isolates from Northwest Ethiopia.

In this study, the GenoType® MTBDRplus assay had a sensitivity of 92% and specificity of 99% for INH resistance, and 100% sensitivity and specificity for RMP resistance and for multidrug resistance. Other reports have shown that the performance of the GenoType® MTBDRplus assay in sensitivity and specificity almost comes up to that of conventional culture-based susceptibility testing: Causse et al. [28] reported a sensitivity of 95% for INH and 100% for RMP, a Meta analysis report by Bawanga et al. [29] showed that GenoType® MTBDRplus assay has a sensitivity and specificity of 96% and 100% for INH and 99% and 99% for RMP, respectively. In the present study, 8% of phenotypically defined isoniazid-resistant strains had no mutations in codon 315 of the katG gene and in the regulatory region of the inhA gene, demonstrating that other mechanisms or mutations in other codons of the katG gene may be responsible for the development of INH resistance in M. tuberculosis strains. Interestingly, all phenotypically defined rifampicin-resistant strains and multidrug-resistant strains had mutations conferring resistance to rifampicin, and both isoniazid and rifampicin resistance (MDR). Suggesting that the set of the DNA probes used in the GenoType® MTBDRplus assay covers most of the mutations prevalent in Northwest Ethiopia. However, previously reported associations between the gene mutations and Beijinger strains [30,31] suggest that the assay may be potentially useful in the area with a high prevalence of Beijinger family (Eastern Europe, China and South-East Asia).

Previous studies have shown that 40-95% of isoniazid resistance are defined as the high-level drug-resistance due to the katG gene mutations. Of which, 75-90% are recognized as mutations in the 315th codon of the katG gene, which mainly result in Ser315Thr and Ser315Thr2 mutation [13,15,32]. In the present study, 94% of INH resistances, close to the high limit of reported range, were attributed to katG mutations which confer high level resistance to INH. Of which, 100% were identified as Ser315Thr1 mutation. Studies have also shown that 8% to 43% of INH resistance are defined as the low-level drug resistance mainly caused by the mutations in the promoter region of inhA gene [33]. In this study, we have observed that the low-level drug-resistance proportion was 6%, close to the low limit of the reported range.

In the previous studies [14,15,34], about 95% of resistance to RMP are associated with the rpoB gene mutations which are found to cluster mainly in the region of codon 507-533. In this study, the distribution of gene
mutation among RMP resistant isolates was 73% at position Ser531Leu and 7% at His526Asp. In 20% of the resistant isolates, mutation was detected only at the wild type probes, which is different from the previously reported gene mutation distribution in China, 37% at Ser531Leu, 3% at His526Asp and in 60% of the isolates, mutation was detected only at the wild type probes [35], reflecting the difference in the distribution of gene mutations associated with RMP resistance in different geographical locations. The high frequency (20%) of RMP resistant isolates with no mutation at the mutant probes, probably indicating the presence of less common mutations at rpoB gene that can not be detected by the current version of the GenoType® MTBDRplus assay.

In this study, the distribution of gene mutation among 8 isolates showing resistance to EMB by GenoType® MTBDRsl assay was 25% at Met306Ile, 13% at Met306Val and 63% of the strains had mutation only at the wild type probes. Furthermore, 58% of the isolates showing resistance to ethambutol by the Bact/ALERT 3D system did not display a similar result by this molecular assay even on repeat assays. Consequently, in the present study, GenoType® MTBDRsl assay had sensitivity of 42% and specificity of 100% for ethambutol resistance. Similarly, other previous studies have shown that this assay has low sensitivity, only about 50% for detection of EMB resistance [36-38]. The present study together with previous reports, highlight the fact that the molecular basis of EMB resistance in *M. tuberculosis* is still insufficiently understood to allow detection of EMB resistance by molecular methods.

**Conclusions**

In our study, the dominance of single gene mutations associated with the resistance to isoniazid and rifampicin in the codon 315 of the katG gene and codon 531 of the rpoB gene was observed. The GenoType® MTBDRplus assay is a sensitive and specific tool for diagnosis of resistance to INH, RMP and MDR. The short turnaround times and the potential for rapid screening of large numbers of isolates make it suitable as a first-line screening assay for TB drug resistance. Its application and popularization will help better solve the long-standing problem of laboratory diagnosis of drug resistance in Ethiopia. In the majority of phenotypically ethambutol resistant isolates, gene mutation associated with the resistance to ethambutol was not detected by this assay. This indicates that the present version of the GenoType® MTBDRsl assay shows limitations in detecting resistance to ethambutol. Further studies are required to understand the mechanism of resistance to ethambutol and to evaluate GenoType® MTBDRplus assay for the diagnosis of INH and RMP resistance from direct sputum specimens of tuberculosis patients in Ethiopia.

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**Authors’ contributions**

BJ was the primary researcher, conceived the study, designed, participated in sample collection, performed laboratory experiments, conducted data analysis and drafted the manuscript for publication. JB participated in doing the laboratory experiments, interpreting the results, and reviewed the initial and final drafts of the manuscript. RE, US and AR reviewed the initial and final drafts of the manuscript. All authors read and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

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Molecular epidemiology and transmission dynamics of *Mycobacterium tuberculosis* in Northwest Ethiopia: new phylogenetic lineages of *Mycobacterium tuberculosis* found in Ethiopia

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Abstract

**Background:** Although Ethiopia ranks seventh among the world’s 22 high-burden tuberculosis (TB) countries, little is known about strain diversity and transmission. In this study, we present the first in-depth analysis of the population structure and transmission dynamics of *M. tuberculosis* strains from Northwest Ethiopia.

**Methods:** 244 Mycobacterium tuberculosis complex (MTBC) isolates where analysed by MIRU-VNTR (mycobacterial interspersed repetitive unit - variable number tandem repeat) 24-loci and spoligotyping to determine phylogenetic lineages and perform a cluster analysis. Clusters of strains with identical genotyping patterns were considered as an indicator for the rate of recent transmission.

**Results:** The population structure was found to be highly diverse: out of the 244 MTBC isolates 59.0% were classified into nine previously described lineages: Dehli/CAS (38.9%), Haarlem (8.6%), Ural (3.3%), LAM (3.3%), TUR (2.0%), X-type (1.2%), S-type (0.8%), Beijing (0.4%) and Uganda II (0.4%). Interestingly, 31.6% of the strains investigated were grouped into four new lineages and were named as Ethiopia_3 (13.1%), Ethiopia_1 (7.8%), Ethiopia_H37Rv like (7.0%) and Ethiopia_2 (3.7%) lineages. The remaining 9.4% of the isolates could not be assigned to the known or new lineages. Overall, 45.1% of the isolates were grouped in clusters, indicating a high rate of recent transmission. Similarly, 66.7% of MDR strains were grouped in clusters.

**Conclusion:** This study confirms a highly diverse MTBC population structure, the presence of new phylogenetic lineages and a predominance of the Dehli/CAS genotype in Northwest Ethiopia. The high rate of recent transmission indicates defects of the TB control program in Northwest Ethiopia. This emphasizes the importance of strengthening laboratory diagnosis of TB, intensified case finding and treatment of TB patients to interrupt the chain of transmission.

**Key words:** *Mycobacterium tuberculosis*, molecular epidemiology, transmission dynamics
Introduction

Despite the existence of anti-tuberculosis drugs for the last 60 years, tuberculosis (TB) continues to be a major threat worldwide. In 2009, WHO estimated the global incidence of TB with 9.4 million cases. Most of the estimated number of TB cases occurred in Asia (55%) and Africa (30%). The 22 high burden tuberculosis countries account for 81% of all estimated cases worldwide (1). Ethiopia ranks seventh among the world’s 22 high-burden tuberculosis countries. The country had 314, 267 TB cases in 2007, with an estimated incidence rate of 378 cases per 100,000 population (2). According to the Ministry of Health hospital statistics data, tuberculosis is one of the leading causes of morbidity, the fourth most common cause of hospital admission, and the second most common cause of hospital death in Ethiopia (3). Additionally, the countrywide anti-TB drug resistance survey conducted in 2005 showed that the prevalence of multidrug resistant TB (MDR, resistance to at least isoniazid [INH] and rifampicin [RMP]) was 1.6% and 11.8% among new untreated and previously treated TB cases, respectively (4). These data show that the TB epidemic is a significant public health threat in Ethiopia, however, detailed epidemiological data assisted by molecular strain typing that pinpoint actual weaknesses in TB control and underlying disease dynamics are not available yet.

Molecular strain typing (genotyping) has contributed significantly to the understanding of TB epidemiology and has helped to improve TB control by providing information on transmission dynamics (5), determining the importance of reactivation versus exogenous reinfection (6), investigating/confirming outbreaks (7), confirmation of laboratory cross contamination (8) and to identify the clonal spread of successful clones, including multi-drug-resistant ones (9). Furthermore, molecular typing has revealed that the MTBC has a diverse population structure with manifold lineages that show large differences in their geographical occurrence and, also, in their pathobiological properties e.g. development and spread of drug resistance (10).

In Ethiopia, few molecular epidemiological studies have been done so far only for the isolates from patients in the capital city, Addis Ababa (11-13). Studies were limited by small sample size, incomplete epidemiological information and/or use of a genotyping method with low strain discriminatory capacity. Recent data are only available from an MDR strain targeted study from year 2006. However, the strains were investigated by spoligotyping only, allowing neither for high resolution phylogenetic strain classification nor for analysis of transmission dynamics (11).

In this study, we used a combination of mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) typing and spoligotyping to investigate a large collection of MTBC
strains isolated from patients living in Amhara region, Northwest Ethiopia. In contrast to classical molecular typing methods such as IS6110 DNA fingerprint and spoligotyping, 24-loci MIRU-VNTR genotyping allows for a high-resolution discrimination of isolates for epidemiological studies and a valid phylogenetic strain classification (14). The data obtained are likely to allow for new insights into population structure and transmission dynamics, thus also revealing urgently needed data to improve TB control in Ethiopia.
Materials and Methods

Study design, area and study period
A total of 260 smear positive pulmonary tuberculosis patients diagnosed at Gondar Hospital, Gondar Health Center, Metemma Hospital, Bahir Dar Hospital and Debre Markos Hospital between March 2009 and July 2009 were included in this study. For all study subjects, information on the socio-demographic data, history of previous tuberculosis treatment, HIV status and the drug susceptibility patterns of the *M. tuberculosis* isolates was available. Informed consent was obtained from the study subjects and the single morning sputum sample and 5ml venous blood sample were collected prior to commencing TB treatment. A structured questionnaire was used to classify patients into new and previously treated tuberculosis cases and to collect socio-demographic data of the study subjects. Institutional ethical clearance was obtained from the research and publication committee of University of Gondar, Ethiopia. Specimens were stored and transported to the Institute of Medical Microbiology and Epidemiology of Infectious Diseases, University Hospital of Leipzig, Germany as described previously (15) for culture and drug susceptibility testing.

Culture and drug susceptibility testing (DST)
Isolation, identification and DST were performed as described previously (16). Briefly, specimens were processed and cultured according to the Deutsches Institut für Normung (DIN) recommendations (17) using Lowenstein Jensen (L-J) media, Gottsacker media and the BacT/ALERT 3D system. Isolates were identified by DNA hybridization technology (GenoType® MTBC; Hain Lifescience, Nehren, Germany) following the manufacturer’s instructions. DST for first line drugs including isoniazid, rifampicin, streptomycin, ethambutol and pyrazinamide was performed by BacT/ALERT 3D system (BioMerieux, S.A , France) according to the methods developed for MB/BacT system (18, 19).
DST for second line drugs including fluorquinolones (FLQ) (ofloxacin & moxifloxacin) and aminoglycosides (AM) /cyclic peptides (CM) (capreomycin, viomycin/kanamycin and amikacin) was performed using DNA hybridization technology on nitrocellulose strips (GenoType® MTBDRsl; Hain Lifescience, Nehren, Germany) following the manufacturer’s instructions. Patients’ serum samples were screened for HIV-1 and HIV-2 using Vironostika HIV Uni-Form II Ag/Ab enzyme-linked immunosorbent assay (ELISA) kit (Bio-Merieux, Boxtel, The Netherlands) following the manufacturer’s instructions.

DNA extraction. DNA was extracted from all isolates by heating mycobacterial pellets obtained from liquid culture, suspended in 200 µL 10 mM Tris-HCl, 1 mM EDTA (pH 7.0) buffer at 95°C for 20
minutes followed by 15 minutes sonication in a sonicating water bath. The suspension was centrifuged at 15,000 rpm for 1 minute, and the supernatant was stored at -20°C until used.

**Genotyping**

All isolates were analyzed by spoligotyping technique as described previously (20) and by 24 - loci MIRU-VNTR genotyping technique as described previously (14). Briefly, for MIRU-VNTR genotyping, 24 loci were amplified by using the MIRU-VNTR typing kit (Genoscreen, Lille, France). Analyses of the PCR products were performed by using the Rox-labeled MapMarker 1,500 size standard (BioVentures, Inc., Murfreesboro, VT) for mix 5 and 1000 size standard (BioVentures, Inc., Murfreesboro, VT) for other mixes (mix 1-4, and mix 6-8), and using the ABI 3130 XL sequencer with 16 capillaries (Applied Biosystems, Foster City, CA). Sizing of the PCR fragments and assignment of the various VNTR alleles were done by using the GeneMapper software version 4.0 (Applied Biosystems, Foster City, CA).

The MIRU-VNTR 24-loci profiles and spoligotyping patterns were used to classify the strains into main phylogenetic lineages by using the reference strain collection and identification tools available online at www.miru-vntrplus.org (21). Briefly, a stepwise identification procedure was carried out as follows. The strains were first classified by the simple match approach that is based on the best match with strains of the reference database. The cut of distance for lineage assignment was set to 0.17. In a second step, phylogenetic tree identification was carried out. Additionally, for each MIRU-VNTR 24-loci pattern a unique MLVA 15-9 code was assigned by using the MIRU-VNTRplus nomenclature.

Cluster analyses of molecular typing data were performed with the Bionumerics software (version 6.6; Applied Maths, Sint-Martens-Latem, Belgium) according to the manufacturers’ instructions. Similarities of genotyping patterns among strains were calculated by using the categorical coefficient. A dendrogram was generated by using the unweighted pair group method with arithmetic averages (UPGMA). Minimum spanning tree analysis was done based on MIRU-VNTR typing data by using the categorical coefficient. For the cluster analysis, a cluster was defined as a minimum of two strains harbouring identical genotype pattern (using composite data, MIRU-VNTR 24-loci and spoligotyping) from different patients belonging to the study subjects. The recent transmission index (RTI) was calculated as (number of clustered patients - number of clusters)/total number of patients. Determination of the discriminatory power of the genotyping methods (MIRU-VNTR 24-loci typing and spoligotyping) was calculated using the Hunter-Gaston Discriminatory Index (HGDI) as previously described (22).
Statistical analysis

All laboratory data were entered, cleared and analyzed using SPSS version 13 statistical package software (SPSS Inc., Chicago, IL). Categorical data were compared by the chi-square test or the fisher exact test, when expected cell sizes (n) were smaller than 5. Two models were constructed in a logistic regression analysis using clusters and anti-TB drug resistance as the respective outcome variables. In order to determine independent risk factors, odds ratios (OR) and 95% confidence intervals (CI) were calculated by using logistic regression analysis for demographic (gender, age, address and religion), epidemiologic (previous treatment and HIV status), and microbiological variables (drug resistance, and infection by *M. tuberculosis* lineages). P-values less than 0.05 were considered statistically significant.
RESULTS

Demographic characteristics

A total of 260 *M. tuberculosis* isolates were utilized to carry out MIRU-VNTR 24-loci and spoligotyping analysis. Out of these, 16 isolates were excluded from the final analysis as for 15 of these, no PCR amplicon was obtained at two or more loci and one multidrug resistant isolate was identified as a mixture of two independent strains during MIRU-VNTR typing. An occasional lack of PCR amplification of some loci has been reported in previous studies (14, 23). This might be explained by chromosomal deletion, nucleotide polymorphisms in the sequences complementary to PCR primers (24), or insufficient DNA quality. A mixture of two independent strains was also defined by the presence of double alleles at two or more loci (14, 25). Isolates with no PCR amplicon at only one locus were treated as missing data at the respective loci and included into the analysis. These observations remained the same even after repeated testing with freshly prepared materials. For the remaining 244 isolates, valid genotyping data were obtained and used for further analyses.

Some demographic data of the study subjects and complete drug susceptibility test results for the isolates used herein were included in our previous report (16). Briefly, the mean age ± the standard deviation of 244 study subjects was 31.6 ± 12.5 (range, 68 years), and 58.2% patients were male. Nearly all patients, 98.8% were Amhara by ethnicity, and 97.5% patients were Christian by religion. Of all study subjects, 17.6% patients were previously treated cases and 25.4% patients were HIV co-infected. Overall, 40 patients (16.4%) had a strain showing resistance to at least one first line drug tested. Thirty-four strains (13.9%) were resistant to INH, 14 (5.7%) strains were resistant to RMP, and 12 (4.9%) were MDR (Table 1). All isolates were susceptible to second line anti-TB drugs tested.

Table 1: Demographic characteristics of the study subjects, drug resistance patterns, phylogenetic lineages and their association with clustering

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total N (%)</th>
<th>Genotyping patterns</th>
<th>OR (95% CI)</th>
<th>p-value</th>
</tr>
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<td>Gender</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Male</td>
<td>142 (58.2)</td>
<td>63</td>
<td>0.9 (0.6-1.6)</td>
<td>0.791</td>
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<tr>
<td>Female</td>
<td>102 (41.8)</td>
<td>47</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Age group (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 45</td>
<td>210 (86.1)</td>
<td>93</td>
<td>0.8 (0.4-1.6)</td>
<td>0.534</td>
</tr>
<tr>
<td>&gt; 45</td>
<td>34 (13.9)</td>
<td>17</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Address</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rural</td>
<td>112 (45.9)</td>
<td>52</td>
<td>1.1 (0.7-1.8)</td>
<td>0.697</td>
</tr>
<tr>
<td>Urban</td>
<td>132 (54.1)</td>
<td>58</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Religion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Christian</td>
<td>238 (97.5)</td>
<td>108</td>
<td>1.7 (0.3-9.2)</td>
<td>0.693*</td>
</tr>
<tr>
<td>Muslim</td>
<td>6 (2.5)</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Previous TB</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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<td>No</td>
<td></td>
<td></td>
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<tr>
<td>---------------------</td>
<td>----------------------</td>
<td>---------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>treatment</strong></td>
<td>43 (17.6)</td>
<td>26</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>2.1 (1.1-4.2)</td>
<td>0.026</td>
<td></td>
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<tr>
<td><strong>HIV status</strong></td>
<td>201 (82.4)</td>
<td>84</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>117</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ISoniazid</strong></td>
<td>62 (25.4)</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resistant</td>
<td>34 (13.9)</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.5 (1.2-5.4)</td>
<td>0.013</td>
<td></td>
<td></td>
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<tr>
<td><strong>Rifampicin</strong></td>
<td>182 (74.6)</td>
<td>86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resistant</td>
<td>210 (86.1)</td>
<td>88</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>122</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Streptomycin</strong></td>
<td>26 (10.7)</td>
<td>10.4 (1.3-84.7)</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>Resistant</td>
<td>12 (4.9)</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.7 (1.5-14.9)</td>
<td>0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ethambutol</strong></td>
<td>218 (89.3)</td>
<td>96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resistant</td>
<td>226 (92.6)</td>
<td>96</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>130</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pyrazinamide</strong></td>
<td>12 (4.9)</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resistant</td>
<td>232 (95.1)</td>
<td>102</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>130</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Resistant to one</strong></td>
<td>40 (16.4)</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>or more FLD</td>
<td>204 (83.6)</td>
<td>85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resistant</td>
<td>25</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.3 (1.2-4.7)</td>
<td>0.015</td>
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<td></td>
</tr>
<tr>
<td><strong>MDR-TB</strong></td>
<td>12 (4.9)</td>
<td>8</td>
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<tr>
<td>Yes</td>
<td>232 (95.1)</td>
<td>102</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>130</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Resistant to all</strong></td>
<td>9 (3.7)</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLD</td>
<td>235 (96.3)</td>
<td>102</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>10.4 (1.3-84.7)</td>
<td>0.012*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>133</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>M. tuberculosis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lineages</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dehli/CAS</td>
<td>95 (38.9)</td>
<td>51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethiopia_3</td>
<td>32 (13.1)</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haarlem</td>
<td>21 (8.6)</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethiopia_1§</td>
<td>19 (7.8)</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethiopia_H37Rv like</td>
<td>17 (7.0)</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethiopia_2</td>
<td>9 (3.7)</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>URAL</td>
<td>8 (3.3)</td>
<td>0</td>
<td></td>
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<tr>
<td>LAM</td>
<td>8 (3.3)</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TUR</td>
<td>5 (2.0)</td>
<td>4</td>
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<td></td>
</tr>
<tr>
<td>X-type</td>
<td>3 (1.2)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-type</td>
<td>2 (0.8)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beijing</td>
<td>1 (0.4)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UgandII</td>
<td>1 (0.4)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not defined</td>
<td>23 (9.4)</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>244 (100.0)</td>
<td>110 (45.1)</td>
<td>134 (54.9)</td>
<td>-</td>
</tr>
</tbody>
</table>

*=Fisher exact test, FLD= First line anti-TB drugs, MDR= Multidrug resistant, §= reference category, LAM= *M. tuberculosis* Latin American Mediterranean
Population structure and cluster analysis

According to the phylogenetic classification of 244 *M. tuberculosis* isolates, 144 (59.0%) were classified into previously described lineages as follows: 95 (38.9%) strains were Dehli/CAS, 21 (8.6%) Haarlem, 8 (3.3%) Ural, 8 (3.3%) LAM (Latin American Mediterranean), 5 (2.0%) TUR, 3 (1.2%) X-type, 2 (0.8%) S-type, 1 (0.4%) Beijing and 1 (0.4%) Uganda II lineage. Interestingly, 77 (31.6%) of the isolates were appear to form four previously undefined lineages, the largest of which having 32 (13.1%) isolates was named Ethiopia_3, followed by a branch with 19 (7.8%) isolates and was named as Ethiopia_1, a third branch with 17 (7.0%) isolates that were closely related to the laboratory strain H37Rv was named Ethiopia_H37Rv like, and the fourth branch with 9 (3.7%) strains was named as Ethiopia_2. The remaining 23 (9.4%) isolates could not be assigned to a known phylogenetic lineage or a new lineage (Fig. 1, Table 1). To confirm this strain classification, we calculated a minimum-spanning tree (MST) based on the MIRU-VNTR 24- loci data. The MST (Fig. 2) confirmed the classification according to UPGMA tree-based analysis (Fig. 1) and by comparison with the MIRU-VNTRplus reference database. All lineages suspected from dendrogram-based analysis were also detected as clonal complexes in the MST, including the newly described genotypes (Fig.2).
FIG. 1. Radial UPGMA tree based on the copy numbers of MIRU-VNTR 24-loci. The tree was calculated by using the MIRU-VNTRplus website. Abbreviations: LAM=\textit{M. tuberculosis} Latin American Mediterranean.
Cluster analysis revealed that 110 of the 244 strains (45.1\%) shared a genotyping pattern with at least one other isolate and were grouped in 36 clusters ranging in size from 2 to 13 strains, resulting in a recent transmission index (RTI) of 30.3\%. The remaining strains were discriminated into 134 unique genotypes. Strains were also assigned to MLVA MtbC15-9 types (Table 2, Fig.S1 in the supplemental material). The largest cluster (n = 13; cluster 5: MLVA MtbC15-9 type 594-15) is formed by the
Ethiopia-3 genotype, followed by the second largest clusters formed by strains of the Dehli/CAS genotype (n = 8, cluster 19: MLVA MtbC15-9 type 1557-32), indicating ongoing transmission of these strains.

Of 12 MDR strains, 8 (66.7%) were in 5 clusters; three clusters contained exclusively 2 MDR strains each, indicating successful transmission of MDR strains within the community, one cluster contained one MDR strain and one INH, EMB and STM resistant strain, and one cluster contained one MDR, one INH and STM resistant strain and three fully susceptible strains, indicating transmission of INH resistant strains that later developed MDR.

Table 2. Discriminatory capacities of spoligotyping and MIRU-VNTR 24-loci, alone or in combination for *M. tuberculosis* isolates from Ethiopia

<table>
<thead>
<tr>
<th>Genotyping method</th>
<th>No. of different patterns</th>
<th>No. of isolates with unique pattern</th>
<th>No. of clusters</th>
<th>No. of isolates in clusters</th>
<th>Clustering rate (%)</th>
<th>RTI (%)</th>
<th>HGDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spoligotyping</td>
<td>69</td>
<td>43</td>
<td>26</td>
<td>201</td>
<td>82.4</td>
<td>64.8</td>
<td>0.88</td>
</tr>
<tr>
<td>MIRU-VNTR 24-loci</td>
<td>161</td>
<td>124</td>
<td>37</td>
<td>120</td>
<td>49.2</td>
<td>34.0</td>
<td>0.97</td>
</tr>
<tr>
<td>MIRU-VNTR 24-loci + Spoligotyping</td>
<td>170</td>
<td>134</td>
<td>36</td>
<td>110</td>
<td>45.1</td>
<td>30.3</td>
<td>0.97</td>
</tr>
</tbody>
</table>

HGDI= Hunter-Gastone discriminatory index, highest degree for HGDI is 1 that shows highest discriminatory power of a method, RTI= Recent transmission index

Factors associated with strain clustering

When the clustering rates were stratified for strains of different phylogenetic lineages, we observed striking differences. The odds of clustering was 21-fold higher among TUR lineage (4 out of 5 strains) (P=0.017), 14-fold higher among Ethiopia_3 lineage (23 out of 32 strains) (P<0.001), 6-fold higher among Dehli/CAS lineage (51 out of 95 strains) (P=0.006) and 6-fold higher among Ethiopia_H37Rv like lineage (9 out of 17 strains) (P= 0.024) compared to Ethiopia_1 linage (3 out of 19 strains) (Table 1).

The odds of clustering was also 2-fold higher among strains from previously treated cases (26 out of 43 strains) (P=0.026) compared to strains from the new cases, nearly 3-fold higher among INH
resistant strains (22 out of 34 strains) compared to INH susceptible strains (P=0.013), nearly 4-fold higher among STM resistant strains (19 out of 26 strains) (P=0.002) compared to STM susceptible strains, and nearly 5-fold higher among EMB resistant strains (14 out of 18 strains) (P=0.004) compared to EMB susceptible strains. Additionally, strains that were resistant to one or more first line anti-TB drugs had 2-fold higher odds of clustering (25 out of 40 strains) (P=0.015) compared to fully susceptible strains, and strains that were resistant to all first line anti-TB drugs had 10-fold higher odds of clustering (8 out of 9 strains) (P=0.012) compared to strains that were susceptible to at least one first line anti-TB drugs. However, multidrug resistance was not a significant risk factor for clustering (8 out of 12 strains) (OR=2.5, P = 0.123) compared to non multidrug resistant strains (Table 1).

Interestingly, more than 50% of MDR strains were classified as the Haarlem lineage (Table 3). Although the numbers are small, the odds of having multidrug resistance was 22-fold higher among patients with a Haarlem strain (P <0.001) compared to patients with the non Haarlem strains and the odds of resistance to all first line anti TB-drugs was 10-fold higher among patients with a Haarlem strain (P=0.004) compared to patients with non Haarlem strains. Similarly, significantly higher risk of resistance to INH (P=0.017), RMP (P<0.001), STM (P=0.015), EMB (P=0.002) or PZA (P=0.002) was observed among patients with a Haarlem lineage compared to patients with the non Haarlem strains (Table 4).
Table 3: Anti-TB drug resistance, patients’ history of previous TB treatment and HIV status stratified for *M. tuberculosis* genotypes

<table>
<thead>
<tr>
<th>M. tuberculosis genotypes (n)</th>
<th>INH N (%)</th>
<th>RMP N (%)</th>
<th>STM N(%)</th>
<th>EMB N(%)</th>
<th>PZA N(%)</th>
<th>MDR N (%)</th>
<th>RFLD N (%)</th>
<th>PT HIV + N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dehli/CAS (95)</td>
<td>10 (10.0)</td>
<td>3 (3.3)</td>
<td>10 (10.5)</td>
<td>7 (7.4)</td>
<td>2 (2.1)</td>
<td>2 (2.1)</td>
<td>2 (2.1)</td>
<td>19 (20.0)</td>
</tr>
<tr>
<td>Ethiopia_3 (32)</td>
<td>10 (31.0)</td>
<td>3 (9.4)</td>
<td>6 (18.8)</td>
<td>5 (15.6)</td>
<td>4 (12.5)</td>
<td>3 (9.4)</td>
<td>3 (9.4)</td>
<td>7 (21.9)</td>
</tr>
<tr>
<td>Haarlem (21)</td>
<td>7 (33.3)</td>
<td>7 (33.3)</td>
<td>6 (28.6)</td>
<td>6 (28.6)</td>
<td>5 (23.8)</td>
<td>7 (33.3)</td>
<td>4 (19.0)</td>
<td>2 (9.5)</td>
</tr>
<tr>
<td>Ethiopia_1 (19)</td>
<td>1 (5.3)</td>
<td>0</td>
<td>1 (5.3)</td>
<td>0</td>
<td>1 (5.3)</td>
<td>0</td>
<td>0</td>
<td>5 (26.3)</td>
</tr>
<tr>
<td>Ethiopia_H37Rv like (17)</td>
<td>2 (11.8)</td>
<td>0</td>
<td>1 (5.9)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3 (17.6)</td>
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<tr>
<td>Ethiopia_2 (9)</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
<td>3 (37.5)</td>
</tr>
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<td>Ural (8)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2 (25.0)</td>
</tr>
<tr>
<td>Tur (5)</td>
<td>2 (40.0)</td>
<td>0</td>
<td>1 (20.0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (20.0)</td>
<td>2 (40.0)</td>
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<td>X-type (3)</td>
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<td>2 (66.7)</td>
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<tr>
<td>Beijing (1)</td>
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<td>0</td>
</tr>
<tr>
<td>UgandII (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Not defined (23)</td>
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<td>1 (4.3)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5 (21.7)</td>
<td>7 (30.4)</td>
</tr>
<tr>
<td>Total (244)</td>
<td>34 (13.9)</td>
<td>14 (5.7)</td>
<td>26 (10.7)</td>
<td>18 (7.4)</td>
<td>12 (4.9)</td>
<td>12 (4.9)</td>
<td>9 (3.7)</td>
<td>43 (17.6)</td>
</tr>
</tbody>
</table>

n= number, RFLD= Resistant to all first line anti-TB drugs, MDR= Multidrug resistant, LAM= *M. tuberculosis* Latin American Mediterranean, PT= previously treated cases, INH=isoniazid, RMP= rifampicin, STM= streptomycin, EMB= ethambutol, PZA= pyrazinamide, + = positive
Table 4: *M. tuberculosis* Haarlem lineage and its association with anti-tuberculosis drug resistance

<table>
<thead>
<tr>
<th>Drug resistance</th>
<th><em>M. tuberculosis</em> lineages</th>
<th>OR (95% CI)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Haarlem (n)</td>
<td>Non Haarlem (n)</td>
<td></td>
</tr>
<tr>
<td>Isoniazid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>7</td>
<td>27</td>
<td>3.6 (1.3-9.8)</td>
</tr>
<tr>
<td>S</td>
<td>14</td>
<td>196</td>
<td>1</td>
</tr>
<tr>
<td>Rifampicin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>7</td>
<td>7</td>
<td>15.4 (4.7-50.2)</td>
</tr>
<tr>
<td>S</td>
<td>14</td>
<td>216</td>
<td>1</td>
</tr>
<tr>
<td>Streptomycin</td>
<td></td>
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</tr>
<tr>
<td>R</td>
<td>6</td>
<td>20</td>
<td>4.1 (1.4-11.6)</td>
</tr>
<tr>
<td>S</td>
<td>15</td>
<td>203</td>
<td>1</td>
</tr>
<tr>
<td>Ethambutol</td>
<td></td>
<td></td>
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<tr>
<td>R</td>
<td>6</td>
<td>12</td>
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</tr>
<tr>
<td>S</td>
<td>15</td>
<td>211</td>
<td>1</td>
</tr>
<tr>
<td>Pyrazinamid</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>5</td>
<td>7</td>
<td>9.6 (2.7-33.8)</td>
</tr>
<tr>
<td>S</td>
<td>16</td>
<td>216</td>
<td>1</td>
</tr>
<tr>
<td>MDR-TB</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>7</td>
<td>5</td>
<td>21.8 (6.1-77.5)</td>
</tr>
<tr>
<td>No</td>
<td>14</td>
<td>218</td>
<td>1</td>
</tr>
<tr>
<td>Resistant to all FLD</td>
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<td></td>
<td></td>
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<tr>
<td>Yes</td>
<td>4</td>
<td>5</td>
<td>10.3 (2.5-41.8)</td>
</tr>
<tr>
<td>No</td>
<td>17</td>
<td>218</td>
<td>1</td>
</tr>
</tbody>
</table>

R = resistant, S = sensitive, n = number, * = Fisher exact test, FLD = First line anti-TB drugs, MDR-TB = multidrug resistant tuberculosis
Discussion

Recent advances in molecular strain typing such as the development of 24-loci MIRU-VNTR typing provide a powerful tool to analyze MTBC population structure and transmission dynamics locally and on the global level, which provides valuable information for the development of effective tuberculosis control policy. In this study we present the first in-depth analysis of the population structure of *M. tuberculosis* strains in Ethiopia based on high-resolution MIRU-VNTR 24-loci typing and spoligotyping. Our data confirm a highly diverse population structure that comprises, thirteen phylogenetic lineages, four of which were not described before. Furthermore, our data indicate a high rate of ongoing recent transmission, of which the spread of MDR strains is of special importance.

Only few in-depth analyses of the population structure of *M. tuberculosis* strains are available from Africa. Most of the investigations are limited by sample size, available epidemiological information, and/or by the genotyping technique used. Previous reports have shown that studies based on spoligotyping only are limited by the inherent problems to perform a valid phylogenetic classification of a large proportion of isolates in a certain area due to high rates of homoplasy (26, 27). On the contrary, the MIRU-VNTR 24-loci typing method applied in our study has the advantage to allow for high-resolution genotyping needed for molecular epidemiological studies and, simultaneously, for valid phylogenetic strain classification enabling screening for new phylogenetic lineages/clonal complexes (14).

Using this method, 90.6% of the strains investigated were classified to *M. tuberculosis* lineages; of which, 58.9% were described before and 31.6 % were newly described in this study. We documented that *M. tuberculosis* Dehli/CAS is the predominant phylogenetic lineage in Ethiopia, accounting 39% of investigated strains. Similarly, a previously published study from the capital city of Ethiopia showed that 43.5% of the strains were CAS genotype (11), and a study from Sudan (28) also showed that *M. tuberculosis* Dehli/CAS is the predominant lineage (49%) of investigated strains. The Dehli/CAS lineage is essentially localized in the Central Asia and Middle-East, more specifically in India (29). Two hypotheses could explain the presence of high Dehli/CAS lineage in Ethiopia: (i) the large Indian and Chinese communities in Ethiopia due to the growing economic partnerships between Ethiopia and the two Asian countries, India and China may have contributed in the introduction of this lineage; or (ii) this lineage could have emerged from Ethiopia and migrated through Asia, this hypothesis is in agreement with the suggestion that East Africa is the origin of *M. tuberculosis* complex species (30).
Additionally, we confirmed the presence of previously undefined phylogenetic lineages named as Ethiopia_3, Ethiopia_1, Ethiopia_H37RV-like and Ethiopia_2 that were clearly defined by tree based, as well as by minimum spanning tree-based analysis. However, comparison with other studies is hampered by the fact that they are mainly based on IS6110 DNA fingerprint and/or spoligotyping analysis hindering a valid analysis of the population structure and standardized comparisons base on MIRU-VNTR nomenclature. Thus, the actual picture of *M. tuberculosis* population diversity in African high-incidence settings is largely incomplete and needs a systematic investigation with phylogenetic useful genotyping methods.

This study also showed a significant association between Haarlem strain infection and multi-drug resistance, resistance to all first line anti-TB drugs and resistance to each first line anti-TB drugs including INH, RMP, STM, EMB and PZA. Similarly, a previous study from Tunisia showed that the Haarlem family genotype has a similar relationship with drug resistance and rapid clonal expansion (31). The association between drug resistance and the Beijing genotype is also well documented in previous studies (32, 33). The association of these genotype families with drug-resistant outbreaks clearly demonstrates their epidemic potential (31, 34). From TB-control point of view, it is relevant to understand whether specific genotype families are overrepresented among drug-resistant cases and, in particular, if these resistant strains are successfully transmitted within the community.

Although the interpretations of clustering results remain somewhat controversial, it is accepted that clustering is a marker for recent transmission (35-37). By using degree of recent TB transmission in a study population, one can estimate the efficacy of the TB control program (35). Both high TB incidence and the current drug-resistance rates in Ethiopia are indicative of defects of the TB control program (2, 4, 16). Supporting this suggestion, we found a high rate of clustering, 45.1% of the total strains investigated. This is in agreement with the previous reports from the capital city of Ethiopia that showed clustering rate of 41.2% (13) and 48.1% (12).

Even more important, we confirm elevated cluster rate in drug resistant strains in general as well as for MDR strains. Similarly, there was a significant association between recent transmission and patients with the history of previous TB treatment, infection with INH resistant strains, STM resistant strains, EMB resistant strains, strains resistant to one or more first line anti-TB drugs and patients with strains resistant to all first line anti-TB drugs. This might be due to the fact that, in Ethiopia there is no culture and drug susceptibility testing facility for routine diagnosis of drug resistance, thus, drug resistant-TB is only diagnosed after prolonged treatment with first-line anti-TB drugs and clinical recognition that
treatment has failed. Treatment of drug-resistant TB with standard first line drugs, instead of a regimen designed according to the resistance pattern has several potential adverse consequences: patients remain on inadequate treatment longer, increasing the risk of treatment failure or death; selection of drug resistant strains and patients remain infectious, promoting transmission to close contacts (38).

These data indicate a successful transmission of drug resistant and MDR strains in the community, a situation that needs to be carefully monitored in the future to determine extensive transmission of resistant strains early enough to avoid more significant problems for TB control as already eminent in several parts of Eastern Europe or South Africa (39, 40).

Interestingly, we present evidence of significant association between recent transmission and the Dehli/CAS, Ethiopia_3, TUR and Ethiopia_H37Rv like strain infections. Similarly, Gagneux et al. have recently proposed that the major \textit{M. tuberculosis} lineages have evolved so as to become adapted to specific host genetic backgrounds and are much more likely to transmit and cause disease among patients of the same ethnicity (41).

In conclusion, our study confirms a highly diverse population structure of \textit{M. tuberculosis}, the presence of phylogenetic lineages that were not described before and a predominance of the Dehli/CAS genotype in the Amhara region, Northwest Ethiopia. Our study also showed a significant association between Haarlem strain infection and resistance to first line anti-TB drugs including multidrug reissuance. The high rate of recent transmission underlines active transmission of \textit{M. tuberculosis} including drug-resistant strains, and consequently the inefficacy of TB control program in the study area. This emphasizes the importance of strengthening laboratory diagnosis of TB including culture and drug susceptibility testing, intensified case finding and treatment of TB patients according to the ongoing DOTS program to interrupt the chain of transmission within the community.

\textbf{Competing interests}

The authors declare that they have no competing interests.

\textbf{Acknowledgements}

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References


Summary

Ethiopia ranks seventh among the world’s 22 high-burden tuberculosis countries. The emergence of drug resistant *M. tuberculosis* strains is one of the most important threats to tuberculosis control program in the country. The high prevalence of HIV, widespread poverty and overcrowding have created an environment which made tuberculosis a formidable threat in Ethiopia. For proper treatment and control of tuberculosis, WHO is recommending countries to expand their capacity for culture and drug-susceptibility testing (DST). However, culture and drug susceptibility testing of *M. tuberculosis* is being done only in the national reference laboratory, in the capital city, Addis Ababa. The successful isolation of *M. tuberculosis* from remote settings requires proper collection, storage and transportation of sputum specimens to reference laboratories. To ensure high yield of positive cultures, WHO recommends that two sputum specimens be collected from remote areas and transported to reference laboratories without delay. There may be a substantial cost associated with handling, transportation and processing of two sputum specimens from each patient. In addition, sputum preservatives, such as 0.6% cetylpyridinium bromide (CPB) or 1% cetylpyridinium chloride (CPC), are recommended when delay is unavoidable. CPC, the most commonly used preservative keeps tubercle bacilli viable only for up to 8 days. Moreover, previous studies have shown the negative effect of CPC on microscopic examination and culture results. Therefore, a simple and inexpensive method for sputum storage that preserves the viability of tubercle bacilli for long periods of time will be useful for proper laboratory diagnosis, epidemiological studies and drug resistance surveillance from remote settings.

Due to the lack of diagnostic facility in the country, drug resistant TB is diagnosed after prolonged treatment with first-line anti-TB drugs and clinical recognition that treatment has failed. Treatment of drug-resistant TB with standard first-line drugs, instead of a regimen designed according to the resistance pattern has several potential adverse consequences: patients remain on inadequate treatment longer, increasing the risk of treatment failure or death; selection of drug resistant strains and patients remain infectious, promoting transmission to close contacts. In order to optimize standard anti-tuberculosis drug therapy and to increase the success of TB control programs, it is important to know the pattern of drug resistance in a country. However, only limited data on the level and risk factors of first line and second line anti-TB drug resistance are available in Ethiopia.

WHO is also recommending countries to consider new, molecular-based assays for the diagnosis of drug resistance because of their high speed, sensitivity and specificity. Molecular methods that have been developed to detect drug resistance include the GenoType® MTBDRplus for detection of isoniazid (INH) and rifampicin (RMP) resistance and the GenoType® MTBDRsl for detection of
resistance against ethambutol (EMB), fluoroquinolones (ofloxacin and moxifloxacin), and aminoglycosides/cyclic peptides (capreomycin, viomycin/kanamycin and amikacin) (Hain Lifescience, Nehren, Germany). The performance of GenoType® MTBDRplus and GenoType® MTBDRsl assays has been studied in several laboratories of other countries. However, there is a wide variation in circulating M. tuberculosis strains worldwide and false negative results reported due to unique genetic mutations, affecting the performance of molecular assays for drug susceptibility testing. Therefore, analysis of gene mutations associated with resistance to anti-tuberculosis drugs and assessment of the performance of molecular methods for drug resistance testing in different settings are needed to ensure acceptable performance of the assays.

Recent advances in molecular technology such as the development of mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) 24-loci typing and spoligotyping methods provide a powerful tool to analyze M. tuberculosis genotypes and transmission dynamics, which should be valuable for development of effective tuberculosis control policy. In Ethiopia limited epidemiological studies have been done so far only for isolates from patients in the capital city, Addis Ababa. Moreover, studies were limited by small sample size, incomplete epidemiological information and use of a genotyping method with low strain discriminatory capacity.

Lack of the aforementioned information makes the tuberculosis prevention and control efforts difficult in Ethiopia. Therefore, this study was done to fill the information gaps that are urgently needed for designing effective tuberculosis prevention and control strategies in the country.

**Publication I:** To investigate the recovery rate of M. tuberculosis from smear positive single morning sputum specimens subjected to long term storage at -20°C, single morning sputum samples were collected from all smear-positive tuberculosis patients diagnosed at Gondar Hospital, Gondar Health Center, Metemma Hospital, Bahir Dar Hospital, and Debre Markos Hospital in Northwest Ethiopia between March and July 2009. Specimens were stored at -20°C at the study sites and sent to the mycobacteriology laboratory at the University Hospital, Leipzig, Germany, where specimens were processed and inoculated into the BacT/Alert 3D system, Lowenstein-Jensen and Gottsacker media. Ice packs were added in the package of the specimens during transport. A total of 319 patients were enrolled in this study. The median specimen storage time was 132 days (range, 16 to 180 days). Of all specimens, 287 (90.0%) were culture positive. The length of time of sputum storage had no significant effect on the recovery rate of M. tuberculosis in all culturing systems. However, lower degree of smear positivity and lower sputum volume (< 2ml) were significant factors for a low recovery rate. In
conclusion, single morning sputum specimens collected at remote settings stored at -20°C for long periods of time without the addition of chemical preservatives can yield a high recovery rate. These findings suggest a simple and cost-effective method of sputum storage for epidemiological and drug resistance studies in low resource countries.

**Publication II:** To assess the level and risk factors for first and second line anti-TB drug resistance, DST for first-line drugs including isoniazid, rifampicin, streptomycin, ethambutol and pyrazinamide was performed by BacT/ALERT 3D system. DST to second-line drugs including fluoroquinolones and aminoglycosides/cyclic peptides was performed using GenoType® MTBDRsl. Of 260 *M. tuberculosis* isolates investigated, 41 (15.8%) were resistant to at least one first-line drug, 13 (5.0%) were multidrug resistant (MDR) and 9 (3.5%) were resistant to all first-line drugs. Any resistance to isoniazid, rifampicin, streptomycin, ethambutol and pyrazinamide was 36 (13.8%), 15 (5.8%), 26 (10.0%), 19 (7.3%) and 12 (4.6%), respectively. MDR-TB was 8/214 (3.7%) among new cases and 5/46 (10.9%) among previously treated cases. All isolates were susceptible to second-line drugs. Previously treated cases and patients diagnosed at Metemma hospital had significantly higher level of drug resistance compared with new cases and patients from Debre Markos hospital, respectively. In conclusion, a substantial number of new and previously treated cases harbour MDR-TB. We recommend DST at least for previously treated cases, patients remain smear positive at the end of the second month of treatment and patients with close contact to MDR-TB cases. Moreover, improved infection control measures need to be implemented in Ethiopia.

**Publication III:** The performance of the GenoType® MTBDRplus for detection of resistance to INH, RMP and MDR and GenoType® MTBDRsl assay for detection of EMB resistance was evaluated compared to the BacT/ALERT 3D system as a reference method, and the frequency of gene mutations associated with resistance to INH, RMP and EMB was analyzed among 260 *M. tuberculosis* isolates. The GenoType® MTBDRplus assay had a sensitivity of 92% and specificity of 99% to detect INH resistance, and 100% sensitivity and specificity to detect RMP resistance and MDR. The GenoType® MTBDRsl assay had a sensitivity of 42% and specificity of 100% for EMB resistance. Of 260 *M. tuberculosis* isolates investigated by the molecular methods, mutations conferring resistance to INH, RMP, or EMB were detected in 35 (13.5%), 15 (5.8%), and 8 (3.1%) isolates, respectively, while mutations conferring resistance to both INH and RMP, multidrug resistance were present in 13 (5.0%) of the isolates. Of 35 INH resistant strains, 33 (94.3%) had mutations in the *katG* gene at Ser315Thr and two strains had mutation in the *inhA* gene at C15T. Among 15 RMP resistant isolates, 11 (73.3%) had *rpoB* gene mutation at Ser531Leu, one at His526Asp, and three strains had mutations but the
Summary

amino acid changes could not be detected by this method. Of 8 EMB resistant strains, two had mutations in the \textit{embB} gene at Met306Ile, one at Met306Val, and five strains had mutations but the amino acid changes could not be detected. In conclusion, the GenoType(R) MTBDRplus assay is a sensitive and specific tool for diagnosis of resistance to INH, RMP and MDR. However, the GenoType(R) MTBDRsl assay shows limitations in detecting resistance to EMB. The dominance of single gene mutations associated with the resistance to INH and RMP was observed in the codon 315 of the \textit{katG} gene and codon 531 of the \textit{rpoB} gene, respectively.

Publication IV: In this study, the first in-depth analysis of the population structure and transmission dynamics of \textit{M. tuberculosis} strains is presented from Northwest Ethiopia. MIRU-VNTR 24-loci typing and spoligotyping methods were used to identify \textit{M. tuberculosis} phylogenetic lineages and to perform cluster analysis. Clusters of strains with identical genotyping patterns were considered as an indicator for the rate of recent transmission. Of 244 \textit{M. tuberculosis} isolates, 144 (59.0\%) were classified into nine previously described lineages: 95 (38.9\%) were Dehli/CAS, 21(8.6\%) Haarlem, 8 (3.3\%) Ural, 8 (3.3\%) LAM, 5(2.0\%) TUR, 3 (1.2\%) X-type, 2 (0.8\%) S-type, 1 (0.4\%) Beijing and 1 (0.4\%) Uganda II lineage. Interestingly, 77 (31.6\%) of the isolates were grouped into four new phylogenetic lineages and were named as Ethiopia_3, 32 (13.1\%); Ethiopia_1, 19 (7.8\%); Ethiopia_H37Rv like, 17 (7.0\%); and Ethiopia_2, 9 (3.7\%). The remaining 23 (9.4\%) strains could not be assigned to the known or new lineages. Patients with Haarlem lineages had 22-fold higher odds of multidrug resistance and 10-fold higher odds for resistance to all first line anti-TB drugs compared with patients infected with non-Haarlem lineages. Overall, 110 (45.1\%) of the isolates were grouped in clusters, indicating a high rate of recent transmission. Similarly, 8/12 (66.7\%) MDR strains were grouped in clusters, indicating successful transmission of MDR strains within the community. In conclusion, this study confirms a highly diverse population structure of \textit{M. tuberculosis}, the presence of new phylogenetic lineages and a predominance of the Dehli/CAS genotype in Northwest Ethiopia. The high rate of recent transmission indicates defects of the TB control program in Northwest Ethiopia. This emphasizes the importance of strengthening laboratory diagnosis of TB, intensified case finding and treatment of TB patients to interrupt the chain of transmission within the community.

In general, this dissertation is of a paramount importance to the tuberculosis prevention and control efforts in Ethiopia as it fills the information gaps that are urgently needed by health professionals for the proper diagnosis and management of tuberculosis patients, and by tuberculosis control program planners for designing effective prevention and control strategies in the country. Furthermore, researchers in the field of mycobacteriology can benefit out of the findings of this dissertation.
References


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Annex I

Fig. Supplemental material 1. Spoligotype patterns and genotype classification of the strains; the strains are ordered in a UPGMA tree based on the similarity of MIRU-VNTR 24-loci patterns.
Annex II

Declaration of Authorship

I hereby declare that the work presented in this dissertation has been designed and performed independently, without help from others and without other materials than stated in the text. To the best of my knowledge and belief, thoughts and ideas from other people and colleagues that have been adopted directly or indirectly in this dissertation were specifically indicated and acknowledged in any case. I confirm that others did not either directly or indirectly receive any payment in kind for any work related to the content of this dissertation. This dissertation has never been submitted before for the award of any other degree or during any kind of examination procedure in any other institution.

Leipzig, 23. 04. 2012

Signature............................

Belay Tessema
Annex III

Curriculum Vitae

Name: Belay Tessema Belay
Date of Birth: April 29, 1980
Place of Birth: Finoteselam, Gojjam, Ethiopia
Nationality: Ethiopian
Language: Amharic, English and German

Educational Background

Primary/Secondary school
1986-1991: Geray primary and junior school, Finoteselam, Ethiopia

University / Collage

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<tr>
<td>2000-2003</td>
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<td>2005-2007</td>
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<td>2008-2012</td>
<td>Doctorate</td>
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<td>University of Leipzig, Germany</td>
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Work experience

1998 - 2000. Technical assistant in the department of Medical Laboratory Technology,
 University of Gondar.

2003 - 2004. Graduate assistant II in the Department of Medical Laboratory Technology, University of Gondar.

2004 - 2005. Assistant lecturer in the Department of Medical Laboratory Technology,
 University of Gondar.

2007. Medical Laboratory expert in CDC/JHPIEGO Ethiopia, National Reference Laboratory,
 Ethiopia.

2007 - 2008. Lecturer in the Department of Medical Laboratory Technology, University of Gondar.
Research Grant awards and Certificates of Merit.

1. 2008. German Academic Exchange Service (DAAD) PhD scholarship award for the project “Molecular Epidemiology and drug resistance of *M. tuberculosis* among HIV positive and HIV negative tuberculosis patients in Northwest Ethiopia”

2. 2008. HIV/AIDS Prevention and Control Office (HAPCO) research Grant award for the project “Magnitude and determinants of nonadherence and nonreadiness to highly active antiretroviral therapy among people living with HIV/AIDS in Northwest Ethiopia.”

3. 2007. Certificate of appreciation and recognition by the University of Gondar “Certificate of appreciation and recognition for his great contributions to the success of academic agreement between University of Gondar, Ethiopia and University of Tokushima, Japan”

4. 2006, United Nations Childrens’ Fund (UNICEF) Research grant award for the project entitled “study on the efficacy of BCG vaccination in preventing *M. tuberculosis* infection among Ethiopian children”

5. 2005 Gondar University Research Grant award for the project “Assessment of the bacteriological quality of milk from diary farms in Northwest Ethiopia”

6. 2003, Jimma University best research award (*Certificate of Merit*)
Annex IV

Publications


22. Belay Tessema, Joerg Beer, Matthias Merker, Frank Emmrich, Ulrich Sack, Arne Rodloff, Stefan Niemann. Molecular epidemiology and transmission dynamics of Mycobacterium tuberculosis in Northwest Ethiopia: New phylogenetic lineages found in Ethiopia. Manuscript has been prepared for submission to the journal of Emerging Infectious Diseases.

Presentations

A. Oral Presentations


B. Poster Presentation
Annex V
Acknowledgements

Four years ago I arrived at Leipzig for the first time with the determination to learn new things and completing a doctorate program. I never imagined that this long-lasting journey would also give me the opportunity to meet such an amazing group of people without whom I would have not survived these intense years. That is why I would like to express my most sincere gratitude to all those people who have made this dissertation possible.

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This is a great opportunity to express my respect and special thanks to Prof. Dr. med. Frank Emmrich, for writing me the letter of admission in the Institute of Clinical Immunology and for his invaluable support, concern and encouragement throughout my work. He has played a great roll to make my long years dream a reality. I also owe my most sincere gratitude to Prof. Dr. med. Arne C. Rodloff, who gave me the unique opportunity to work in the Institute of Medical Microbiology and Epidemiology of Infectious Diseases with excellent research facilities and under the supervision of highly qualified staff. He has always found time to listen my questions, to discuss on problems and to find the best solutions. He has also supported me to find a laboratory to perform the genotyping in Borstel. Without his generous support and encouragement it would have been difficult to accomplish this work.

I would like to sincerely thank Dr. Stefan Niemann, not only for giving me the opportunity to perform the genotyping experiments at Molecular Mycobacteriology, research centre, Borstel, Germany, but also for his professional guidance and support during the experiments and analysis of the genotyping data. My deep gratitude also goes to Elisabeth Krawczyk for her motherly care and kind assistance during my training and throughout my lab-work. I would also thank Tanja Ubben for her assistance during genotyping by MIRU-VNTR typing and spoligotyping methods, Julia Zallet for her assistance during spoligotyping and Matthias Merker for his assistance during analysis of genotyping data using bionemetics software. I also give my sincere appreciation to all data collectors and study participants from all study areas in Ethiopia.
This study was carried out with the financial support from Fraunhofer Institute for Cell Therapy and Immunology, Leipzig, Germany; Institute of Medical Microbiology and Epidemiology of Infectious Diseases, University Hospital of Leipzig, Germany; German Academic Exchange Service (DAAD), Germany and University of Gondar, Ethiopia.

I would like to express my heartfelt gratitude to Prof. Dr. Dieter Reissig for his fatherly support, advice, concern and care from the time of getting admission letter from Prof. Dr. med Frank Emmrich until the end of my study. I am also truly indebted and thankful for his tireless and long standing efforts to improve the quality of education, health service provision and research activities at the University of Gondar through qualified and educated staffs. Long live Prof. Dr. Dieter Reissig!

I gratefully appreciate my colleagues; Antneh Amsalu, Lamesgin Muluken, Gizachew Yismaw, Ebba Abate, Yeshambel Belyhun, Belay Anagaw, and Afewerk Kassu for their encouragement and support during sample and data collection time. I would also love to thank my colleagues in Leipzig and their families; Yemataw Wondie, Abebe Muche, Kahsay Huruy, Netsanet Worku, Andargachew Mulu, Fantahun Biadglene, Tigist Alemu and Temesgen Fufa whose warm hospitality, encouragement, sharing experiences and ideas helped me a lot. Especially, the most fun and joyful events we had: Friday evening parties, holidays and many other occasions made me feel at home.

I am deeply indebted to my parents in Heaven, for raising me with love, splendid care and encouragement to live a purpose-driven life. My brothers, my sisters and your families, thank you very much for your sincere love, concern, care and support in many ways. I own sincere gratitude to my father in-law and my mother in-law for their constant prayers and encouragement. I would also like to thank my brother in-laws, my sister in-laws and their families for their encouragement and support.

My wife, Shewaye (Enateyee), I can never thank you enough for your crucial comments throughout this work, love, care, support, encouragement and understanding. You are the source of inspiration and the reason of success in my life. I love you forever! My little child, Surafel, I love you so much! God bless you!

Finally and most importantly, Almighty God, thank you for giving me favour in your sight. You always have a key for every problem, a light for every shadow, a relief for every sorrow and a plan for every tomorrow. Thank you!