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Department of Microbiology, Immunology and Parasitology

Molecular epidemiology and drug-resistance pattern of *Mycobacterium tuberculosis* isolates from Amhara, Gambella and Benishangul-Gumuz regions of Ethiopia

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List of Abbreviation/acronyms

Am	Amikacin
APHI	Amhara Public Health Institute
BD	Beckton Dickinson
Cm	Capreomycin
DNA	Deoxyribonucleic acid
DST	Drug Susceptibility Testing
EMB	Ethambutol
EPHI	Ethiopian Public Health Institute
IRB	Institutional Review Board
Eto	Ethionamide
GC	Growth Control
INH	Isoniazid
Km	Kanamycin
Lfx	Levofloxacin
LPA	Line Probe Assay
Mfx	Moxifloxacin
MGIT	Mycobacterium Growth Indicator Iube
MTBC	*Mycobacterium tuberculosis* complex
MDR TB	Multidrug resistant tuberculosis
NTRL	National Tuberculosis Reference Laboratory
Ofx	Ofloxacin
PCR	Polymerase Chain Reaction
PZA	Pyrazinamide
RIF	Rifampicin
SIT	Spoligotyping International Typing
STR	Streptomycin
SPSS	Statistical Package for Social Sciences
TB	Tuberculosis
TIC	Treatment Initiative Centre
WHO	World Health Organization
WT	Wild-type

Abstract

Background: Globally, tuberculosis (TB) has affected human beings for thousands of years and the emergence of drug-resistant tuberculosis has been a major obstacle to control the disease, especially in developing countries including Ethiopia.

Objective: The study investigated the molecular epidemiology and drug resistance pattern of *M. tuberculosis* circulating in the Amhara, Gambella and Benshangul-Gumuz regions of Ethiopia.

Methods: A cross-sectional study was conducted using 128 MTBC isolates that were obtained from presumptive TB/MDR-TB patients. A total of 127 recovered isolates were used to investigate drug resistance patterns with the indirect proportion method using the MGIT 960 system and the genotypic method at the Ethiopian Public Health Institute. Further, identification of mycobacterial strain types and mycobacterial lineages was done using spoligotyping. Data were managed using the Epi-info, SPSS version 25, SITVIT2, MIRU-VNTRplus and TBInsight databases.

Results: Of the 127 recovered mycobacterial isolates, 100 (78.7%) were sensitive to the four first-line anti-TB drugs, but 27 (21.3%) were resistant to atleast one anti-TB drug, 20 (15.7%) were poly-resistant, 17 (13.4%) were multi-drug resistant and 2 (10.5%) were Pre-XDR. The highest gene mutations associated with INH and RIF resistance were observed in the *katG MUT1* gene (S315T1) [20 (76.9%)] and *rpoB* gene (S531L) [10 (52.6%)], respectively. Further, the two pre-XDR cases showed mutations in the *gyrA* gene. Among the 127 spoligotyped isolates, 119 generated 43 different spoligotyping patterns; 87 (73.1%) generated 26 distinct spoligotype patterns; and 31 (26.0%) generated 17 different spoligotype patterns. The predominantly identified strains, family, lineages and sublineages were the SIT149 (19, 15.9%), T family (54, 45.4%), Euro-American (72, 60.5%) and CAS1-Delhi (24, 20.2%), respectively. Additionally, *Mycobacterium Africanum* (12, 10.1%) was identified. Furthermore, drug resistance was significantly associated with previous TB history (X^2=46.59; p<0,001) and previous TB treatment outcome (X^2=47.677; p<0.001).

Conclusion: The study identified a significant proportion of drug resistant TB (monoresistance, MDR, Pre-XDR) which could imply the drug resistant TB is a major public health problem in the country. Although a diversified mycobaterium lineages were observed in this study, the Euro American lineage and East-African-Indian lineages were the predominant lineages with the respective CAS1-Delhi and T3-ETH sublineages that are strongly associated with the drug-resistant TB. Consequently, the country should focuse on the strategies that help for early detection and treatment of drug-resistant TB to halt the transmission of the disease. We recommend further molecular characterization of the unknown mycobacterial isolates, particularly using sequencing platforms and phenotypic drug susceptibility testing to first- and second-line drugs. Besides, the country's tuberculosis control program should monitor patient adherence to ensure the patients complete the full course of treatment.

Key terms: Drug resistance pattern, Ethiopia, genetic diversity, Molecular epidemiology, *Mycobacterium tuberculosis*, Spoligotyping

1. Introduction

1.1. Background information

Tuberculosis (TB) is a disease of broad host range, caused by the *Mycobacterium tuberculosis* complex (MTBC) and remains a major public health problem (Daniel *et al*.,1994; Delogu *et al*., 2013; World Health Organization, 2020). Despite a slow decline in the incidence of TB in recent years, millions of people continue to fall sick with TB each year. According to the World Health Organization (WHO) report of 2020, TB is the 10th leading cause of death and the leading cause from single infectious agents above HIV/AIDS globally (World Health Organization, 2020).

Globally, there is an estimate of 10.0 million new TB cases and 1.2 million TB deaths among HIV-negative people with 208,000 additional deaths from TB among HIV-positive people in the year 2019 (World Health Organization, 2020). Global TB rates are falling at a slow pace. The trend has been noted for a slight increase in Multidrug resistant TB (MDR-TB) cases as a proportion of all TB cases with the burden of MDR-TB either increasing faster or decreasing more slowly than the overall TB burden in each reporting country (World Health Organization, 2018).

Drug-resistant TB continues to be a public health crisis worldwide. In 2019, there were an estimated 465,000 new cases of rifampicin-resistant TB (RR-TB) which is resistant to the most effective first-line drug rifampicin and of these, 78% had MDR-TB globally (World Health Organization, 2020). According to the 2020 global TB report, the majority of MDR/RR-TB cases are from the top 30 highest TB countries. According to the same report, Ethiopia is among the 30 high TB burden countries with an estimated incidence of 140 (95% CI: 98–188) TB cases and 1.3 (95% CI: 0.87_1.8) MDR/RR-TB cases per 100,000 population (World Health Organization, 2020). There were an estimated 23,800 deaths due to TB, including HIV-related deaths in Ethiopia during the same period. The majority of the estimated MDR-TB cases remain undiagnosed because of limitations in diagnostic services and hence will continue to spread drug-resistant strains in the community (World Health Organization, 2018; Grobusch *et al*., 2018).

The global TB epidemic consists of multiple genotype-specific sub-epidemics resistant to the first-line anti-TB drugs at the molecular level. To avoid further spread of the disease, early detection of drug-resistant TB is critical (Fox *et al*., 2017). The most common approach to investigate the burden of drug-resistant TB in resource-limited settings is through surveys conducted every 5

years. Even though the coverage of drug sensitivity testing (DST), which is essential to estimate MDR and extensively drug-resistant TB (XDR-TB), is generally improving, the ability to diagnose MDR and XDR-TB is limited in developing countries including Ethiopia (World Health Organization, 2018; Grobusch MP. and Kapata N., 2018). The global incidence of MDR/XDR-TB is viewed only as an estimate, which implies that the actual burden might be underestimated (World Health Organization, 2018).

Nowadays, one of the critical global health problems is the emergence and spread of drug-resistant TB including MDR-TB (resistance to at least rifampicin and isoniazid), pre-extensively resistant TB (pre-XDR-TB) (MDR plus resistance for one fluoroquinolone) and XDR-TB (MDR plus resistance to any fluoroquinolone and at least one additional Group A drugs [levofloxacin, moxifloxacin, bedaquiline and linezolid]) (World Health Organization, 2021). The increasing use of GeneXpert and the treatment of RR-TB with MDR-TB regimens without considering resistance to other drugs has increased the number of reported RR-TB cases in the 30 high MDR-TB countries. In this connection, XDR-TB continues to account for 9% of RR/MDR-TB cases and has been also reported by Ethiopia (World Health Organization, 2020).

Molecular characterization of *M. tuberculosis* strain in the community contributed to recognize the epidemiology of TB and provided transmission dynamics information and improved the control of the disease (Tessema *et al*., 2013). Molecular typing revealed a diverse population structure with various lineages of the MTBC with large differences in their geographical occurrence and pathogen biology e.g. development and spread of drug resistance (Mathema *et al*., 2006).

Tuberculosis control policies have targeted the prevention of drug-resistant tuberculosis through the WHO directly observed treatment short course (DOTs) strategy and focused on the detection of drug-resistant tuberculosis of high-risk groups with a history of prior treatment for active tuberculosis (Falzon *et al*., 2017). In addition to the diagnosis of TB, improving drug-resistance detection is an essential component of effective TB control and TB patient management for combating the disease.

Specific strains of *M. tuberculosis* belonging to distinct phylogenetic clusters (lineages) may differ in virulence, pathogenesis, and epidemiologic characteristics, which may significantly impact strategies of TB control and vaccine development (Gagneux *et al*., 2007; Coscolla *et al*., 2014). Additionally, knowledge of the molecular epidemiology of drug resistant tuberculosis is helpful in supporting countries' tuberculosis surveillance and control programs. However, the molecular

epidemiology data of drug-resistant TB is still scanty especially in resource limited regions of Ethiopia, with only a few studies performed in selected regions of the country as reviewed recently (Tulu *et al*., 2018). Moreover, the genetic diversity studies on *M. tuberculosis* from Ethiopia have demonstrated the presence of new mycobacterial lineages like lineage 7/Aethiops vertus (Firdessa *et al*., 2013; Nebenzahl-Guimaraes *et al*., 2016), Ethiopia 2 and Ethiopia 3 (Tessema *et al*., 2013). This could suggest the presence of unknown mycobacterial lineages within the study areas. Hence, it is meaningful to analyze the drug resistance pattern and molecular epidemiology of *M. tuberculosis* strains in Ethiopia. Therefore, this study investigated the molecular epidemiology and drug resistance pattern of *M. tuberculosis* strains circulating in the Amhara, Gambella and Benshangul-Gumuz regions of Ethiopia.

1.2. Statement of the problem

Despite a slow decline in the incidence of tuberculosis (TB) in recent years, it continues to be a major public health problem globally. One of the barriers to TB control programs globally is the emergence of drug-resistant TB which also becomes a major public health problem. In 2019, 3.3% MDR-TB prevalence in new cases and 17.7% in previously treated cases were detected and reported globally (World Health Organization, 2020). Also, drug-resistant strains often continue to transmit, circulate and persist since full DST profiling and individualized therapy are rarely done (Weyer *et al*., 2017).

Ethiopia is still one of the 30 high TB and MDR-TB burden countries in the world having an estimated incidence of 140 (95% CI: 98–188) TB cases and 1.3 (95% CI: 0.87_1.8) MDR/RR-TB cases per 100,000 populations (World Health Organization, 2020). Ethiopia has an estimated 1,400 MDR-TB patients occurring annually among notified TB cases and has also reported 1 laboratory-confirmed cases of XDR-TB in 2019 (World Health Organization, 2020). However, the majority of the estimated MDR-TB cases remain undiagnosed because of limitations in diagnostic services and hence will continue to spread drug-resistant strains in the community (Weyer *et al*., 2017). This emerging threat of MDR-TB could threaten the recent gains in TB control.

A meta-analysis on Sub-Saharan African countries showed that the risk of developing drug-resistant TB to at least one anti-TB drug was about 3 times higher in individuals who had a previous history of anti-TB treatment than new TB cases (Berhan *et al*., 2013). The risk of having MDR-TB in the previously anti-TB treated TB cases was 5-fold higher than that of the new TB cases. Resistance to Ethambutol and Rifampicin was more than fivefold higher among the previously treated with anti-TB drugs. Similarly, six out of 23 studies reported a high prevalence of MDR-TB in the range of 3.3%-46.3%, and two of them reported XDR-TB in the range of 1% - 4.4% in Ethiopia (Biadglegne *et al*., 2014) and previous exposure to anti-TB drug treatment is the most powerful predictor for the emergence of MDR-TB in Ethiopia. From a study comprising 118 samples from Felege Hiwot Referral hospital, new strains of MTBC were found in 11.1% of isolates which were not registered on the SpolDB database (Debebe *et al*., 2014). Another study in Northwest Ethiopia on 244 isolates reported 9.4% of the isolates to have had no lineage classification (Tessama *et al*., 2013). A study on 92 isolates from a community-based survey of Ethiopia also confirmed 91 of the isolates to have shown good spoligotype patterns with 41 different spoligotypes; however, 65.8 % (27/41) of these were not previously reported (Getahun

et al., 2015). Also, a systematic review from Ethiopia on 21 studies with a total of 3067 strains, identified five lineages including 7.1% Indo-Ocean, 0.2% East Asian/Beijing, 23.0% East African-Indian, 64.8% Euro-American and 4.1% Ethiopian (Tulu *et al*., 2018). On the same systematic review, Euro-American was the most frequently (64.8%) occurring Lineage while East Asian was the least (0.2%) frequently occurring Lineage in the country and the Ethiopian Lineage seemed to be localized to northeastern Ethiopia (Tulu *et al*., 2018). A review conducted by Tulu *et al* found the five top clades as T-48.0%, CAS-23.0%, H-11.0%, Manu-6.0% and Ethiopian-4.1% (Tulu *et al*., 2018). The same review also identified the predominant shared types of SIT149 (420 isolates), SIT53 (343 isolates), SIT25 (266 isolates), SIT37 (162 isolates), and SIT21 (102 isolates) and also, 15% orphan strains.

Although different studies have shown significant genetic variation among TB in the population of Amhara region, there is limited data to show the molecular epidemiology and drug resistance patterns of tuberculosis in Gambella and Benshangul-Gumuz regions (Tessama *et al*., 2013; Tulu *et al*., 2018; Getahun *et al*., 2015). Even the molecular epidemiology studies previously done in different parts of the country revealed diversification of the *M. tuberculosis* strains, where new MTBC lineages were found especially in the Amhara region (Tessama *et al*., 2013). Besides, the transmission dynamics of drug-resistant TB in Ethiopia are not well understood. Therefore, it will be meaningful to update the information on the drug resistance pattern in Amhara region and investigate the situations in the selected study areas (Gambella and Benshangul-Gumuz regions), where molecular epidemiological study is extremely limited.

1.3. The significance of the study

Molecular epidemiology is a powerful approach to monitor infectious disease and uses a multidisciplinary approach to identify the factors that determine cause, spread and distribution of disease (Mathema *et al*., 2006; Savine *et al*., 2002). Moreover, by comparing isolates from different geographic areas, the current study can provide unique information on the transmission and dynamics of *M. tuberculosis* and allows us to analyze evolutionary changes of the pathogen population and strain lineages (Brosch *et al*., 2002; Mathema *et al*., 2006; Savine *et al*., 2002; Supply *et al*., 2001). Molecular epidemiological findings from developed countries often show high polymorphism in the genetic patterns of *M. tuberculosis* complex (MTBC) strains (Bauer *et al*., 1998; Van Soolingen *et al*., 1999). Rapid methods of detecting infection and the drug susceptibility pattern of the strains circulating in the community are also essential for effective TB prevention and control programs so that further transmission can be halted (Uddin *et al*., 2018).

With the thought that MTBC most likely emerged from the Horn of Africa and spread throughout the world with the migration of human (Brosch *et al*., 2002; Sharma *et al*., 2016), it is possible that the present study might add new information for further identification of lineages which were not described before in the specified regions of the country. Further, investigation of the genotype cluster with spoligotyping could provide previously unrecognized domestic transmission of drug-resistant TB in these regions of the country. Therefore, the current study could provide additional information on the drug resistance pattern and molecular diversity of the MTBC to regional health bureaus and the Ministry of health to facilitate and adopt evidence-based reliable preventive and control strategies. It will also provide crucial information to halt further spread of the disease and to prevent amplification of drug-resistant TB to severe forms due to inappropriate treatment, poor case management and lack of knowledge. Early detection, treatment and establishment of quality service delivery system in hard-to-reach areas will greatly contribute to the country's TB prevention and control program. Moreover, the data from this study will be used as baseline information for further exploration. Furthermore, knowing the molecular epidemiology and drug resistance pattern of *M. tuberculosis* could support the End TB strategy by providing detailed information on the mycobacterial strains of a specific community, which could inter support the search of effective new diagnostic methods, resistance detection tools, the relevance of introducing new drugs, and treatment regimens.

2. Literature review

2.1. Bacteriological characteristics and Classification of *Mycobacterium tuberculosis* Complex

Tuberculosis (TB) is an infectious disease caused by the *Mycobacterium tuberculosis* complex (MTBC) that has affected humans and animals since ancient times and was identified by Robert Koch in 1882 (Daniel *et al*., 1994; World Health Organization, 2020). Hence, the MTBC is an aerobic, rod-shaped, nonmotile, spore-free, slow growing microorganism and has a generation time of 12-24 hours under optimal conditions, which is a longer time than that of many bacteria (Delogu *et al*., 2013). Mycobacteria have a unique cell wall structure that provides a strong impermeable barrier to compounds and drugs. The organism is resistant to alcohol and acid; hence refered acid fast bacilli. Acid fastness of the mycobacteria is due to mycolic acid and other complex lipopolysaccharides in the cell wall (van Ingen *et al*., 2017).

Mycolic acid, cord factor and Wax-D are the lipid fractions of the mycobacterial cell wall that perform various functions of bacteria (Goren *et al*., 1972). Mycobacterial mycolic acids are strong hydrophilic molecules that form a protective layer around mycobacteria to affect the permeability properties. The cord factor of mycobacteria also provides a long and rod format for the microorganism. It is the most critical factor for bacterial survival in the host cell and shows a virulent behavior towards mammalian cells. The Wax-D fraction of the mycobacteria helps bacteria to escape from the phagocytic cell or macrophage (Goren *et al*., 1972; Campbell., 1976).

Genetic studies on tuberculosis provided information regarding the classification of mycobacteria. The *Mycobacterium tuberculosis* complex (MTBC) comprises classical species members like: *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium canettii*, *Mycobacterium caprae*, and *Mycobacterium pinnipedii* (Brosch *et al*., 2002). *Mycobacterium tuberculosis* is the primary causative agent of TB disease in humans, and *Mycobacterium bovis* is responsible for bovine TB; and is the second most common cause of TB in humans. *Mycobacterium africanum* is the causative agent of TB in West Africa and affect both humans and animals; causes disease that is clinically indistinguishable from that of *M. tuberculosis* (Brosch *et al*., 2002; Sharma *et al*., 2016). From a taxonomic point of view, they are closely related to *Mycobacterium microti* which is a pathogen of voles and rarely infecting humans (Delogu *et al*., 2013). *Mycobacterium tuberculosis subspecies caprae* are those bacteria that are recently classified into *Mycobacterium caprae* species that show relatively similar characteristics

with MTBC (Aranaz *et al*., 2003) and *Mycobacterium pinnipedii*, which is accepted as the seal bacillus and infects seals and sea-lions but is also isolated from goats (Cousins *et al*., 2003).

Mycobacterium canettii is a genomically diversified tubercle bacilli which has closely related genomic relationship with the most common ancestors of MTBC (Brosch *et al*., 2002) and has evolved by clonal expansion into the MTBC (Brosch *et al*., 2002; Supply *et al*., 2013; Van Soolingen *et al*., 1997). It is characterized by smooth and glossy colonies on culture media, less virulence and also isolated in small patients from or with connections to the horn of Africa (Fabre *et al*., 2004; Gutierrez *et al*., 2005; Van Soolingen *et al*., 1997; Koeck *et al*., 2011).

Additionally, the MTBC comprises of seven phylogenetic lineages for which humans are the only known host. These lineages are known as "human-adapted" MTBC because they have ability to maintain complete cycle of infection, disease and airborne transmission only on humans and has geographical structure from which initial *M. tuberculosis* lineages were derived (Gagneux *et al*., 2018). The phylogenetic lineages includes; the Indo-Ocean (lineage 1), East-Asian (lineage 2), Indian and East-African (lineage 3), Euro-American (lineage 4), West-African 1 (lineage 5), West-African 2 (lineage 6) and Ethiopian (lineage 7) (Brudey *et al*., 2006; Riojas *et al*., 2018). Recently, two lineages that seems restricted to eastern Africa were reported; a new sister lineage that was asigned as "Lineage 8" (Ngabonziza *et al*., 2020) and five isolates that were assigned as "Lineage 9" (Coscolla *et al*., 2021) .

2.2. Drug-resistant Tuberculosis

Drug-resistant TB (DR-TB) remains to be a significant challenge in TB treatment and control programs worldwide. The 2020 global TB report revealed, there were 3.3% MDR-TB prevalence in new cases and 17.7% in previously treated cases globally (World Health Organization, 2020). The distribution and prevalence of MDR-TB continued to be a serious challenge for TB control in Ethiopia despite the relative decline in the incidence of MDR-TB. According to a systematic review on 22 articles, 527 had MDR-TB with the overall prevalence of MDR-TB in all TB cases estimated to be 1.4% from a total of 3849 patients studied (Asgedom *et al*., 2018). The prevalence of MDR-TB ranged from 0 to 46.3%. In Ethiopia, the average mean rate of MDR-TB was found to be 12.6 ± 15.9%. Previous exposure to anti-tuberculosis treatment was the most commonly identified risk factor of MDR-TB in Ethiopia.

From 2006 to 2014 the overall MDR-TB prevalence in Ethiopia showed a stable time trend. Furthermore, a systematic review and meta-analysis that was done using 16 articles revealed an overall prevalence of MDR-TB among newly diagnosed was 2% (95% CI 1% - 2%) and among previously treated TB patients was15% (95% CI 12% - 17%) (Eshetie *et al*., 2017). The same study also revealed previously treated TB patients were significantly associated with the MDR TB infection ($P < 0.001$) and there was an odds ratio of 8.1 (95% CI 7.5–8.7) for previously treated TB patients to develop an MDR-TB infection compared to newly diagnosed cases.

Additionally, a cross-sectional study on 86 isolates from 105 smear-positive pulmonary TB patients from central Ethiopia, reported any drug resistance to one of the four drugs in 23.3% of the isolates (Tilahun *et al*., 2018). In the same study, the highest proportion of any resistance was observed against isoniazid (9.3%) and ethambutol (7%) and a single case (1.2%) of multidrug-resistant (MDR) TB was reported.

2.3. Molecular Epidemiology of Drug-resistant Tuberculosis

Drug-resistant TB (MDR-TB, RR-TB, and XDR-TB) strains of *M. tuberculosis* are man-made problems, mainly related to the lack of quality drugs and poor case management. *M. tuberculosis* complex uses several strategies to resist the action of antimicrobial agents (Nguyen *et al*., 2016). Globally in 2019, there were 206,030 MDR/RR-TB cases, of these 177,099 were enrolled on the second-line TB treatment regimen (World Health Organization, 2020). MDR-TB was reported in 3.3% of new TB cases and 17.7% of previously treated cases globally.

Even though, the drug resistance of TB is increasing in Ethiopia as indicated in different reports , the 2020 WHO TB report indicated that Ethiopia had MDR-TB prevalence of about 0.71% (0.62-0.80) and 16% (14-17) in the new and previously treated TB cases, respectively (World Health Organization, 2020). In the same report, WHO estimated that about 2,700 MDR/RR-TB cases occur among notified TB patients in Ethiopia. Even though the country has reported only 3 laboratory-confirmed cases of XDR-TB in 2019, the majority of the estimated MDR/RR-TB and XDR-TB cases could be undiagnosed due to poor access to diagnostic services.

The molecular epidemiology study from Northwest Ethiopia by Tessema and his colleagues classified 59% of isolates (of 244 isolates) 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%) (Tessema *et al*., 2013). Additionally, 31.6% of the strains

from the same study 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. In the same study, the remaining 9.4% of the isolates could not be assigned to the known or new lineages. Furthermore, 45.1% of the isolates in this study were grouped in clusters, indicating a high rate of recent transmission.

Another study using 92 isolates from a community-based survey of Ethiopia confirmed all of them as *M. tuberculosis* by RD9-based PCR and 91 of them showed good spoligotype patterns with 41 different spoligotypes (Getahun *et al*., 2015). In this study, spoligotype revealed higher diversity (45%), and 65.8% (27/41) among them were not previously reported and also the strains were grouped into 14 clusters consisting of 2–15 isolates and among them the dominant strains were SIT53, SIT149, and SIT37 consisting of 15, 11, and 9 isolates, respectively. This study also revealed 70% (64/91) clustered strains and only 39.1% (25/64) occurred within the same Kebele. The study also showed that 74.7% (68/91) belonged to Euro-American lineage, 18.6% (17/91) to East Africa Indian lineage and the remaining 6.5% (6/91) belonged to Indo-oceanic lineage (Getahun *et al*., 2015). The drug susceptibility test results were available for 90 isolates from the same study; mono-resistance was observed in 27.7% (25/90) while poly-resistance was detected in 5.5% (5/90) of the isolates. Moreover, multi-drug resistance (MDR-TB) was detected in 4.4 % of the isolates whilst the rest 66.7% (60/90) were susceptible to all drugs. In the same study, the highest level of mono-resistance 26.6% (24/90) was observed for streptomycin and the majority (91.1%) of streptomycin mono-resistant strains belonged to the Euro-American lineage.

Another research from central Ethiopia on 86 isolates from 105 smear-positive pulmonary TB patients showed that the majority (76.7%) of the *M. tuberculosis* isolates were susceptible to all the four first-line drugs (Tilahun *et al*., 2018). In the same study, the majority (76.7%) of the isolates were clustered into seven groups while the rest (23.3%) appeared unique. The SIT53 and SIT149 Spoligotypes consisted of 24.4% and 20.9% of the isolates, respectively. In this study, the isolates were assigned to the family using SPOTCLUST software and 45.3%, 23.3%, 13% of them belonged to T1, T3 and CAS family respectively (Tilahun *et al*., 2018).

2.4. Laboratory Diagnosis of Tuberculosis and drug-resistant Tuberculosis

Diagnosis of drug resistance TB is one of the essential steps in the management of tuberculosis. To meet this requirement several methods have been developed, these are conventional (absolute concentration, resistance ratio, and proportion), BACTEC MGIT system, molecular methods etc.

(Mshana *et al*., 1998). However, these methods have some limitations. More rapid methods like BACTEC are very expensive for routine use in high TB endemic and low economic countries. In general, it is best to perform molecular testing on sputum or another specimen before initiating treatment whenever drug resistance is suspected (Seaworth *et al*., 2017). Conventional methods that use solid media (Lowenstein-Jensen media or 7H10 Middlebrook agar) take 6 to 9 weeks to obtain the result. Therefore, simple, rapid and relatively cheap methods are preferred particularly for low-income countries. Detailed descriptions of commonly used diagnosis methods of tuberculosis; molecular and conventional methods testing are presented in the next sub-sections.

2.4.1. Molecular techniques in the diagnosis of tuberculosis and determination of drug-resistance

Commonly, drug-resistant tuberculosis strains arise through the sequential accumulation of resistance mutations for individual drugs (Ramaswamy and Musser, 1998). Therefore, to detect drug resistant *M. tuberculosis,* technologies which can detect mutation on specific genes is required. For this, molecular diagnostic tests based on DNA amplification have been proposed to complement the conventional bacteriological diagnosis of TB (Lin and Desmind., 2014). Most of these tests allow the rapid detection of *M. tuberculosis* and are highly specific although their sensitivity often remains sub-optimal (Miotto *et al*., 2018). Molecular testing for rifampicin resistance is >95% sensitive whereas approximately 80 to 85% sensitive for drugs other than rifampicin (Seaworth *et al*., 2017). Most of Ethambutol resistant isolates can be identified by molecular testing of mutations at the *embB* locus; whereas, all mutations in *embB* are not associated with Ethambutol resistance (Seaworth *et al*., 2017; Lin and Desmind., 2014; Zhang and Yew., 2015). Ethambutol resistance can also be missed on liquid media. There is no significant problem whenever the MTBC isolate is susceptible to all first-line drugs except Ethambutol; however, it leaves only two drugs, rifampicin, and PZA, in the standard first-line regimen when it is associated with INH resistance (Lin and Desmind., 2014). PZA resistance can be detected with molecular testing with most of the PZA mutations found at *pncA* locus (Whitfield *et al*., 2015; Ramirez-Busby and Valafar, 2015); however, some resistant strains do not have *pnc*A mutations. Although some MTBC isolates with mutations associated with fluoroquinolone resistance are detected at the *gyrB* or another locus, primarily fluoroquinolone resistance is identified by mutations in the *gyrA* locus (Miotto *et al*., 2018). Whereas, the MTBC isolates with mutations associated with second line injectable anti-TB agent resistance can be detected at the *rrs* locus (Miotto *et al*., 2018).

Occasionally, molecular testing identifies a mutation consistent with true resistance even though the phenotypic testing does not identify resistance. Brief discussion on commonly used molecular testing methods and their performances is given in the sub-sections hereunder.

2.4.1.1. GeneXpert MTB/RIF assay

The GeneXpert MTB/RIF assay is the most recent addition to the landscape in the molecular detection of *M. tuberculosis* and developed by Cepheid (CA, USA) together with the Foundation for Innovative New Diagnostics (FIND) (World Health Organization, 2011). It is designed for use as a point-of-care TB diagnostic test for simultaneously detection of *M. tuberculosis* and rifampin resistance and is a fully automated system, closed cartridge-based system, semi-quantitative PCR-based system which requires minimal manipulation of sample and operator training and strongly recommended by WHO, particularly for individuals who have the difficulty of producing sputum samples like children and HIV-coinfected individuals (World Health Organization, 2011; Steingart *et al*., 2013). The GeneXpert system has been validated in the USA for the detection of *M. tuberculosis* and rifampicin (RIF) resistance in approximately two hours. This method is based on polymerase chain reaction (PCR) amplifying specific regions of the (*rpoB* gene) and mutation at the rpoB locus; it is >95% sensitive and specific for true rifampin resistance. Furthermore, it does not require advanced infrastructures since all reagents are available as disposable cartridge that is single-use (World Health Organization, 2008).

2.4.1.2. Line Probe Assay (LPA)

Line probe assay (LPA) is a family of DNA strip-based test which determines the drug resistance of *M. tuberculosis* and endorsed by WHO since 2008 as a policy statement (World Health Organization, 2008). It is a combination of the multiplex PCR technology, followed by reverse hybridization and used to identify *M. tuberculosis complex* and detect the drug resistance of first and second-line agents (World Health Organization, 2008; World Health Organization, 2016; World Health Organization, 2016; Global Laboratory Initiative, 2014). The two genotypic kits of Hain Lifescience (Nehren, Germany); MTBDRplus and MTBDRsl assays both of which target on the *23S rDNA*, can be performed both from smear and culture positive specimens. Here is the brief description of the two assays.

The MTBDRplus assay used to identify MTBC and predict the susceptibility or resistance to the two main first-line anti-tuberculosis drugs; RMP and INH. The assay screens for the absence

and/or presence of "wild-type" (WT) and/or "mutant" (MUT) DNA sequences within specific regions of three genes: *rpoB*, *katG* and *inhA* which are associated with RMP resistance, high/low-level INH resistance, respectively (Hain Lifescience, 2012; Kebede *et al*., 2017; Global Laboratory Initiative, 2014). Each strip of the MTBDRplus assay contains 27 reaction zones (bands). The reaction zones include the six control bands (conjugate control (CC), amplification control (AC), *M. tuberculosis complex* control (TUB), *rpoB* control , *katG* control and *inhA* control), eight bands for *rpoB* wild-types and four bands for *rpoB* mutant probes, one band for *katG* wild-type and two bands for *katG* mutant probes and also two bands for *inhA* wild types and four bands for *inhA* mutant probes (Hain Lifescience, 2012; World Health Organization, 2016). Genotype MTBDRplus VER 2.0 assay shows sensitivity, specificity, PPV and NPV of 96.4 %, 100%, 100% and 96.9%, respectively (Ramaswamy *et al*., 1998; Miotto *et al*., 2018).

Currently, the only rapid molecular test recommended by WHO for predicting susceptibility or resistance to second-line anti-TB agents is MTBDRsl assay (World Health Organization, 2016). Furthermore, MTBDRsl assay is used to identify MTBC and predict the susceptibility or resistance to second-line anti-TB drugs (Fluoroquinolones (FQs) and second-line injectable drugs) in addition to identifying MTBC (Hain Lifescience, 2015; World Health Organization, 2016). This assay screens the absence and/or presence of "wild-type" (WT) and/or "mutant" (MUT) DNA sequences within specific regions of the genes; *gyrA*, *gyrB*, *rrs* and *eis* promoter regions which are associated with resistance to resistance to either fluoroquinolones or second-line injectable drugs (World Health Organization, 2016; Hain Lifescience, 2015). This test is normally only available in centralized laboratories due to its requirement for sophisticated laboratory infrastructure and equipment.

2.4.1.3. Spoligotyping (Spacer-Oligotyping)

Spoligotyping is a method which was developed by Kamerbeek and his colleagues in 1997 for the simultaneous detection and typing of MTBC. This method is based on polymerase chain reaction (PCR) amplification of a highly polymorphic direct repeat (DR) locus in the *M. tuberculosis* genome (Kamerbeek *et al*., 1997). The method is a simple, cheap, rapid and reproducible testing method which is used to study the phylogeny of *M. tuberculosis* complex strains (Kremer *et al*., 1999). The rapidity of spoligotyping in detecting both causative bacteria and in providing epidemiologic information on strain identities determine the clinical usefulness of the method. Implementing such a method in clinic settings would be useful in the surveillance of tuberculosis

transmission and in interventions to prevent further spread of this disease but it would not be easy to implement in resource-limited setup. It can also be used to associate phenotypic characteristics of isolates with the genotype family of the bacteria (De Jong *et al*., 2009). However, discrimination level of spoligotyping is generally low and it is necessary to be cautious when using this method to examine the TB transmission at strain level (Kremer *et al*., 2005). The SITVIT database; one of the largest publicly available databases on the *M. tuberculosis* complex, global spoligotype database and included 7,105 spoligotypes from more than 62,582 isolates representing 105 countries can be used for detection and data analysis of spoligotyping (Demay *et al*., 2012; Aminian *et al*., 2010).

2.4.2. Conventional techniques for diagnosis of tuberculosis and drug-resistance

2.4.2.1. Smear Microscopy

Smear microscopy is the most widely used diagnostic method for diagnosis of active pulmonary TB and universally used in resource-limited countries (Global Laboratory Initiative, 2010). The technique relies on the specific lipid-rich cell wall of mycobacteria which prevents access to common aniline dyes but retains the carol-fuchsin or fluorochrome dye (the first dye of staining) after alcohol acid decolorization. *M. tuberculosis* is often known as "acid-fast bacillus (AFB)" because of its unique cell wall structure (Talip *et al*., 2011). Smear microscopy is simple, inexpensive and efficient in detecting pulmonary tuberculosis that are most infectious. The intrinsic reliance on sputum production is the main drawback of sputum smear microscopy in addition to the low sensitivity, and limit its use in children and HIV positive patients who may have paucibacillary disease with fewer AFB, can't expectorate and give a proper sample. Ziehl-Neelsen and fluorescent staining are the two types of acid fast stains; used to detect mycobacteria in sputum specimens using smear microscopy (World Health Organization, 2011).

Sputum smear examination by light microscopy remains the most rapid and least expensive method for directly detecting mycobacteria in clinical specimens and is highly specific. The detection limit of Ziehl-Neelsen stain is around 5000-10000 bacilli/ml of sputum; with overall sensitivity of 20-80% (World Health Organization, 2011). Fluorescent microscopy (FM) is more sensitive and faster, but less specific alternative using acid-fast fluorochrome dyes instead of Ziehl-Neelsen staining. The WHO recommended initiating implementation of fluorescent microscopy with a light emitting diode (LED) particularly in high-load laboratories for TB diagnosis in 2011 (World Health Organization, 2011).

2.4.2.2. *In Vitro* Culture

In vitro culture remains the gold standard method which uses both macroscopic and microscopic characteristics together with the rate of growth and pigmentation for the identification of TB. It is more sensitive than microscopy; detects approximately 10^2 viable organisms/ml of the specimen, and thus can provide a definitive diagnosis for tuberculosis (Global Laboratory Initiative, 2014). Moreover, the viable bacilli from cultured growth can be used for species identification and drug susceptibility testing (DST) (Global Laboratory Initiative, 2014). Sputum samples must be decontaminated before culture inoculation to prevent overgrowth of other faster-growing microorganisms. Culture can take weeks to get result due to very slow growth nature of *M. tuberculosis* with a doubling time of about 20 hours (depending on the growth medium used) (Wang., *et al*, 2014). Generally, two culture systems are available for *M. tuberculosis*; liquid media and solid media. To prevent the overgrowth of normal respiratory flora, antibiotics can be added on the culture media (Global Laboratory Initiative, 2014).

The solid mycobacterial cultures generally need 6 to 8 weeks due to a slow-growing characteristics of organism. The commonly used solid culture medias include Lowenstein-Jensen, Middle-brook agar, Ogawa) (Global Laboratory Initiative, 2014). It allows information like colony counts and visualization of colony morphology. It is used to directly determine whether the MTBC isolate is pure or is mixed with other organisms.

The liquid mycobacterial culture (broth) systems include the commonly used methods like; Mycobacteria Growth Indicator Tube (MGIT) by Becton Dickinson and, to a lesser degree, the BacT/ALERT® by bioMérieux and VersaTrek® by Trek Diagnostic Systems. The MGIT culture system contains a modified Middlebrook 7H9 broth with a fluorescence quenching-based oxygen sensor (silicon rubber impregnated with a ruthenium pentahydrate). Growth is detected by exposing an inoculated MGIT to ultraviolet light and examining for fluorescence, which is an indication of growth and oxygen consumption. The performance of this culture system is mostly equal and in combination with conventional solid media is satisfactory. Liquid media increases the primary isolation of mycobacteria by 10% over solid media (Global Laboratory Initiative, 2014). It is highly recommended to use a combination of solid media and liquid media to ensure good recovery of mycobacteria for subsequent species identification and drug susceptibility testing.

2.4.2.3. Phenotypic drug susceptibility and resistance testing

The susceptibility testing of *M. tuberculosis* has developed along very different lines and now involves very different methodologies. It should be performed in laboratories with extensive experience, particularly for testing second-line drugs against *M. tuberculosis*. Phenotypic DST is a method performed on initial isolates from culture-positive TB patients (Global Laboratory Initiative, 2014). There are three accepted methods for drug susceptibility testing of *M. tuberculosis*: the absolute concentration method, the resistance ratio method and the agar proportion method, the latter being used most widely in the Western hemisphere.

Even though the conventional phenotypic drug susceptibility testing (DST) of *M. tuberculosis* isolates using the Lowenstein-Jensen medium (the proportion method) is considered the gold standard, it takes 4–6 weeks to report results (Global Laboratory Initiative, 2014). For rapid detection of drug resistance in *M. tuberculosis* WHO endorsed the commercial liquid media-based culture systems. Clinical tuberculosis laboratories around the world replaced the BACTEC 460TB system with Mycobacterium Growth Indicator Tube (MGIT) 960 system with similar turnaround time.

2.5. Treatment of drug-susceptible tuberculosis

The discovery of streptomycin in 1943 and the discovery of rifampicin in 1963, led to the effective treatment practice against *M. tuberculosis*. The combination of drugs to prevent the selection of resistances and application of an extended treatment that ensures not only the cure but also the prevention of a possible relapse are the bacteriological basis to be met by any treatment (World Health Organization, 2017; Federal Ministry of Health, 2013). Current WHO guidelines recommend a 6-month treatment regimen consisting of a combination of 4 drugs (Rifampicin, Isoniazid, Ethambutol, and Pyrazinamide) for 2 months and then 2 drugs (Rifampicin and Isoniazid) for 4 months (World Health Organization, 2017). This regimen gives curative rates of up to 90% in immuno-competent (HIV negative) TB patients. For this to be achieved this regimen has to be prescribed at the correct dose taken regularly by the patient and for the required length of time to prevent relapse and development of drug-resistant strains. The drugs in the current regimens are efficient bactericidal compounds at standard or higher doses (for Ethambutol) especially for actively growing bacteria. At lower doses or for slowly multiplying Mycobacteria (Isoniazid) they can be bacteriostatic. Pyrazinamide can be bactericidal or bacteriostatic depending on the concentration reached at the site of infection. The ability of the most commonly used TB

regimens to kill the slowly growing or slowly metabolizing bacteria (i.e. persisters) after rapidly growing or metabolizing bacteria have been killed via bactericidal activity is suboptimal. This is the main reason for the long duration of the current TB treatment regimens. Generally, the first-line anti-TB drugs (FLD) include isoniazid, streptomycin, rifampicin and ethambutol; and there are three injectable second-line drugs known as (amikacin, capreomycin, and kanamycin) (Federal Ministry of Health, 2013).

The most important determinant for the success of therapy is the development of drug resistance. The known targets for each of the four first-line drugs and the genetic location of mutations that confer drug resistance are already known. However, not all mutations that confer resistance have been fully elucidated. *M. tuberculosis* isolates that are resistant to both isoniazid and rifampicin are defined as multidrug-resistant TB (MDR-TB). Resistance to isoniazid and rifampicin plus any fluoroquinolone and at least one of three injectable second-line drugs (amikacin, kanamycin, or capreomycin) is defined as extensively drug-resistant TB (XDR-TB) (Federal Ministry of Health, 2018).

2.6. Treatment of drug-resistant tuberculosis

Drug-resistant TB, especially rifampicin resistance, has become the main challenge in the attempt to eliminate TB from the worldwide. The most active drug against *M. tuberculosis* is Rifampicin. More extensive treatments (at least 18 months) is required for TB cases in which it is not possible to use Rifampicin either due to resistance or intolerance, two conditions that are more difficult to cure and where their prognosis is the poorest. The MDR-TB treatment shall include at least five drugs, including PZA, if the isolate is shown or believed to be susceptible during the initial intensive phase of treatment (World Health Organization, 2020). Regimens with five likely effective drugs, including PZA, resulted in a mortality rate similar to that of a regimen of five likely effective drugs without PZA (World Health Organization, 2020).

WHO has recommended the use of second-line injectable drugs (SLID) for 8 months in the initial or intensive phase of the MDR-RB treatment regimen and effective three or four drugs should be added in the continuation phase with 24 to 26 months of therapy depending on prior exposure for second-line TB drugs. Therefore, second-line TB drugs can be given for prolonged periods which lasts from 18 to 24 months or for a longer duration (World Health Organization, 2020; Federal Ministry of Health, 2018).

Designing treatment regimens for patients with MDR-TB poses several challenges, complicated by a limited choice of second-line drugs, with greater toxicity and less efficacy (Federal Ministry of Health, 2018). At least four second-line anti-TB drugs should be used on the intensive phase for effective MDR-TB treatment. Certain drugs with no clear evidence about the effectiveness can be part of the regimen without considering the success (World Health Organization, 2020; Federal Ministry of Health, 2018). MDR regimens should include at least pyrazinamide, a fluoroquinolone, an injectable anti-TB drug, ethionamide (or prothionamide) and either cycloserine or PAS (para-aminosalicylic acid); and Ethambutol is not recommended to be included in second-line regimens in Ethiopia. A newly diagnosed MDR-TB patients shall receive 8 Z-Cm6-Lfx-Pto (Eto) - Cs at the intensive phase and 12Z-Lfx-Pto (Eto)-Cs at continuation phase (Federal Ministry of Health, 2013; Federal Ministry of Health, 2018).

3. Objectives

3.1. General objective

The main objective of this study was to investigate the molecular epidemiology and drug resistance patterns of *M. tuberculosis* strains circulating in the Amhara, Gambella and Benshangul-Gumuz regions of Ethiopia.

3.2. Specific objectives

- To characterize the phenotypic drug-resistance patterns of *M. tuberculosis* isolates from the three regions of Ethiopia
- To characterize the genotypic drug-resistance patterns of *M. tuberculosis* isolates from the three regions of Ethiopia
- To determine the gene mutation patterns associated with drug-resistant *M. tuberculosis* isolates
- To investigate the molecular epidemiology of *M. tuberculosis* isolates
- To assess the association of drug-resistant *M. tuberculosis* with the geographic location, age, sex, previous TB/MDR-TB history, TB treatment outcome and *M. tuberculosis* lineages

4. Materials and Methods

4.1. Study area

The study was conducted at Ethiopian Public Health institute using the isolates from Amhara, Gambella, and Benishangul-Gumuz regions of Ethiopia. The isolates were isolated from 10 hospitals with high patient flow within the respective regions (8 from Amhara, 1 from Gambella and 1 from Benishangul-Gumuz). The hospitals includes Assosa general hospital, Ataye hospital, Borumeda hospital, Debre Birhan referral hospital, Debre Tabor general hospital, Debre Markos referral hospital, Finote Selam hospital, Gambella hospital, Gonder University hospital and Metema hospital.

4.2. Study Design and Period

A cross-sectional study was conducted at National Tuberculosis Reference Laboratory (NTRL) of Ethiopian Public Health Institute on stored MTBC isolates. The study used MTBC isolates that were obtained from the project entitled "Validation of OMNIgene® SPUTUM for liquefaction, decontamination, and preservation of pulmonary specimens in Ethiopia" which took place from April 2017 to December 2018. A total of 913 sputum samples from consecutive presumed TB/DR-TB patients were enrolled in the project. Further laboratory analysis was conducted on 128 culture confirmed *M. tuberculosis* isolates between January 2019 and March 2020.

4.3. Participant Inclusion and Exclusion Criteria

4.3.1. Inclusion Criteria

- All culture-positive isolates from presumptive TB/DR-TB patients with complete history that were MTBC

4.3.2. Exclusion criteria

- Culture-positive isolates that were Nontuberculous mycobacteria (NTM)

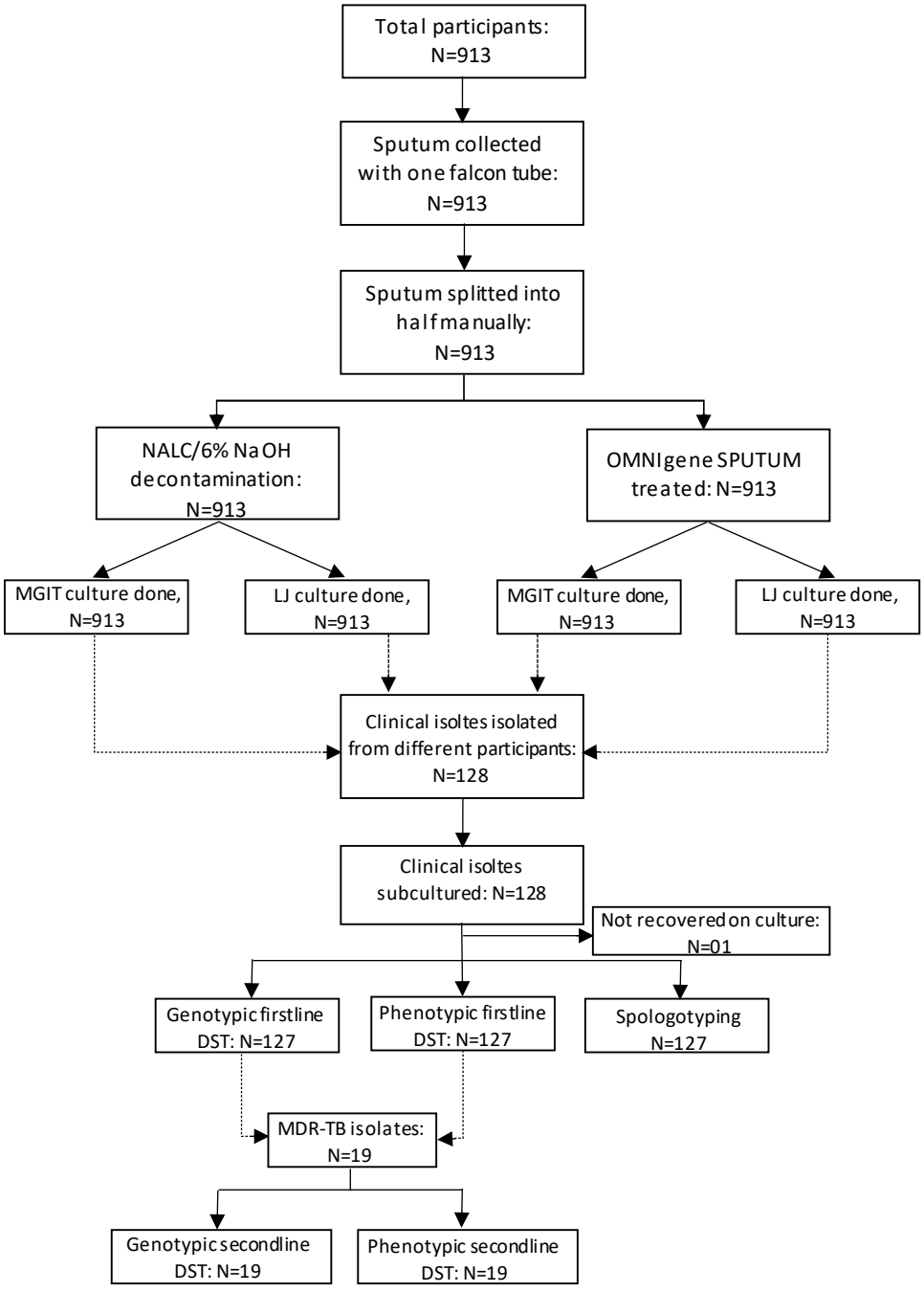

***Figure 1*:** Flow chart showing the laboratory procedures of the study. The culture isolation was done from 913 presumtive TB/MDR TB patients for another study "Validation of OMNIgene® SPUTUM for liquefaction, decontamination, and preservation of pulmonary specimens in Ethiopia" at Amhara Public Health institute and Ethiopian Public Health Institute. The current study was the continuation of the prior study that started by subculturing the 128 mycobacterial isolates for further laboratory analysis.

4.4. Study Variables

4.4.1. Dependent variable

The dependent variables of this study included the drug resistance pattern of *Mycobacterium tuberculosis* complex (MTBC) isolates, gene mutation patterns of MTBC and spoligotyping pattern of MTBC.

4.4.2. Independent variables

The independent variables of this study included demographic variables (age, sex) and clinical characteristics including clinical history, previous exposure of first-line anti-TB drugs (>1 month), previous TB treatment outcome, contact history with a known TB patient, contact history with known MDR-TB patient, co-infections like diabetes mellitus, and HIV.

4.5. Data collection and management

Demographic and clinical data which were collected using a well-structured clinical data collection form (Annex 10.2) were used in this study. All information collected and the laboratory results of different tests were entered in a specifically designed data entry template using Epi-info 2011 version 3.5.4 and the study unique identifier was used to link all the information. All data were double entered to facilitate the data validation. The entered and cleaned data from the Epi-info database were exported to SPSS version 25 for statistical analysis. The paper-based data were kept at EPHI in a locked cabinet and electronic data are protected by a password.

4.6. Laboratory methods

Mycobacterial Sub-culturing

The frozen suspension of the MTBC isolates were thawed at room temperature and subcultured on liquid culture using BD BACTEC MGIT 960 system to perform indirect proportional DST and molecular characterization (Global Laboratory Initiative, 2014). MGIT 960 system uses a liquid nutrient-rich medium and MGIT growth supplement that allow mycobacteria to grow. It also uses a cocktail of antibiotics (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin) that inhibit the growth of other bacteria. The growth of mycobacteria is indicated by fluorescence, that increases proportionally as oxygen decreases in the medium tube. The MGIT instrument detects this fluorescence in the medium using UV light and complex computer algorithms. The subculture was done as follows briefly; the frozen MTBC isolate suspension thawed, thoroughly

vortexed, growth supplement (0.8 ml) without PANTA added into a new MGIT tube aseptically, inoculum diluted with saline solution (1:100), 0.5 ml of specimen (1:100 diluted) inoculated, incubated at 37^{o}C in the MGIT 960 instrument and then monitored until it becomes positive. For the subculture we used supplement (0.8 ml) without PANTA, as the purity test of the isolates was carried out during mycobacterial identification. Mycobacterial isolates were stored after they were confirmed to be free of contamination and minimal contamination is expected during subculture.

Phenotypic drug-susceptibility testing

Phenotypic drug susceptibility testing (pDST) for first-line and second-line anti-TB drugs was performed using BD BACTEC MGIT 960 system following manufacturer's instructions and WHO recommendations (Global Laboratory Initiative, 2014; World Health Organization, 2018). Here, the procedure is briefly described as follows. Lyophilized antibiotic drugs of MGIT 960 SIRE and PZA kit of first-line anti-TB drugs (BD) were reconstituted in sterile distilled water and used for testing; Streptomycin (STR) [1.0µg/ml], Isoniazid (INH) [0.1µg/ml], Rifampicin (RIF) [1.0µg/ml], Ethambutol (EMB) [5.0µg/ml] and Pyrazinamide (PZA) [100µg/ml]. Furthermore, second-line anti-TB drugs (Sigma) were used for susceptibility testing of MDR-TB isolates; Amikacin (Am) [1.0µg/ml], Capreomycin(Cm) [2.5µg/ml], Clofizimine(Cfx) [1.0µg/ml], Ethionamide (Eto) [5.0µg/ml], Kanamycin (Km) [2.5µg/ml], Lenizolide (Lz) [1.0µg/ml], Levofloxacine (Lvx) [1.0µg/ml], Moxifloxacin (Mfx) [0.25µg/ml] and Ofloxacin (Ofx) [2.0µg/ml]. A drug-free growth control tube was included for each isolate. The BD BACTEC MGIT 960 system's software algorithm determines the relative growth ratio between the drug-containing tube and the growth control (GC) tube when the GC tube reaches 400 growth units, and then the device automatically reports the qualitative results as susceptible or resistant. *Mycobacterium tuberculosis* H37Rv strain (ATCC 27294) was tested as a positive control for each batch of the DST run; to maintain the quality of the test.

Deoxyribonucleic acid (DNA) Isolation

The genomic DNA of the isolates were extracted from positive MGIT tubes and tested using the GenoType MTBDR*plus* assay, GenoType MTBDR*sl* assay and spoligotyping. For genotypic DST, the DNA was extracted using GenoLyse kit of Hain Lifescience (Hain Lifescience, 2012; Hain Lifescience, 2015), whereas for spoligotyping, the DNA was obtained by heat treatment of the isolate suspension (Zwadyk et al., 1994). Briefly, the DNA isolation for Spoligotyping was carried out as follows; the 1 ml suspension of liquid culture isolate was heated at 80^{o}C for 45 minutes using a heat block, followed by sonication for an additional 15 minutes. The heat-killed

isolate suspension was centrifuged at 13,000 rpm for 10 minutes at +4°C, and the supernatant (DNA) was collected into a new tube and stored at -20°C for molecular characterization.

GenoType® MTBDRplus assay

The genotypic drug-resistance identification to RIF (based on mutations in *rpoB* gene) and INH (based on mutations in *katG* and *inhA* genes) for all of the 127 isolates was performed using the Genotype® MTBDR*plus* assay at the National Tuberculosis Reference Laboratory following manufacturer's instructions (Hain Lifescience, 2012). The assay was performed indirectly using culture positive isolates. The procedure had DNA extraction and amplification of target sequences with biotinylated primers (AM-A and AM-B), and DNA reverse hybridization steps. The test result was interpreted as per the WHO's recommendations by considering the presence or absence of wild-type (WT) and mutant band on a strip (Global Laboratory Initiative, 2018). The absence of a WT band or presence of mutant band indicates resistance of MTB to an associated drug.

GenoType® MTBDRsl assay

Genotypic drug-resistance identification to Fluoroquinolones (based on mutations in the *gyrA* and *gyrB* genes) and injectable drugs (based on mutations in the *rrs* gene) was performed for the 19 MDR isolates using the Genotype® MTBDRsl assay following manufacturer's instructions (Hain Lifescience, 2015). Briefly, the Genotype® MTBDR*sl* assay was performed from culture positive isolates at the National Tuberculosis Reference Laboratory. The procedure had DNA extraction, amplification of target sequences with biotinylated primers, and DNA reverse hybridization. The test result was interpreted as per the WHO recommendations by considering the presence or absence of wild-type (WT) and mutant band on a strip (Global Laboratory Initiative, 2018). The absence of a WT band or the presence of mutant band indicates resistance of MTB to an associated drug.

Spoligotyping (Spacer-Oligotyping)

All MTBC isolates were genotyped using spoligotyping as described elsewhere (Kamerbeek *et al*., 1997). The direct repeat (DR) region was amplified by a Thermal Cycler using oligonucleotides and primers derived from this region. The primers used were DRa (GGTTTTGGGTCTGACGAC, biotinylated at 5') and DRb (CCGAGAGGGGACGGAAAC). The reaction mixture was amplified with PCR; the mixture was heated for 3 min at 96°C and subjected to 20 cycles of 1 min at 96°C, 1 min at 55°C, and 30 sec at 72°C and 5 min at 72°C and then the amplified product was hybridized to a set of 43 immobilized oligonucleotides. Each oligonucleotide corresponds to one of the unique

spacer DNA sequences within the DR locus. The hybridized DNA was detected by the enhanced chemiluminescence and exposure to X-ray film following the instructions of manufacturer. The hybridization patterns were converted into binary and octal formats and compared with previously reported strains in the SITVIT2 and MIRU-VNTRplus databases (Demay *et al*., 2012; Weniger *et al*., 2010).

4.7. Laboratory quality control

Molecular grade water and *Mycobacterium tuberculosis* H37Rv (ATCC 27294) were included as negative and positive controls, respectively, in every batch of genotypic DST; and only *Mycobacterium tuberculosis* H37Rv (ATCC 27294) was included as a positive control in every batch of phenotypic DST. *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* (AF 2122/97) strains were used as positive controls and molecular grade water was used as a negative control for every batch of spoligotyping.

4.8. Operational Definitions

Multidrug-resistant tuberculosis (MDR-TB): is *Mycobacterium tuberculosis* Complex resistant to at least both isoniazid and rifampicin.

Pre-extensively drug-resistant TB (Pre-XDR-TB): is *Mycobacterium tuberculosis* Complex resistant to both isoniazid and rifampicin plus resistant to any of Fluoroquinolone.

Presumptive TB: A tentative diagnosis of TB that will be confirmed or excluded by subsequent testing. Cases should not remain in this category for more than three months.

New case: A newly registered patient who wasn't on anti-TB treatment for more than a period of one month.

Previously treated case: A newly registered TB patient who have been treated with anti-TB drugs for a period of one month or more. Chemoprophylaxis was not considered as a treatment for TB.

Poly-resistant tuberculosis: is *Mycobacterium tuberculosis* Complex resistant to more than one first-line anti-TB drug, other than both isoniazid and rifampicin.

Mono-resistant tuberculosis: is *Mycobacterium tuberculosis* Complex resistant to one first-line anti-TB drug only.

Extensively drug-resistant TB (XDR-TB): is *Mycobacterium tuberculosis* Complex resistant to both isoniazid and rifampicin plus resistant to any of Fluoroquinolone and at least one additional Group A drug (levofloxacin, moxifloxacin, bedaquiline and linezolid).

4.9. Data analysis

The Epi-info entered data were exported to SPSS version 25 for statistical analysis. The SITVIT2 (the updated version of SITVITWEB) at (http://www.pasteur-guadeloupe.fr:8081/SITVIT2/) and MIRU-VNTRplus at (https://www.miru-vntrplus.org/MIRU/index.faces) databases were used for the analysis and interpretation of Spoligotype results (Demay *et al*., 2012; Weniger *et al*., 2010). Families, subfamilies and variants were assigned using the model-based program of "Spolclust". Lineages were assigned based on spoligotype Large Sequence Polymorphism (LSPs) using an online tool of "TBInsight" at (http://tbinsight.cs.rpi.edu/publication_tblineage.html) (Aminian *et al*., 2010; Shabbeer *et al*., 2012). Descriptive analysis; frequencies, proportions were used to explain the demographic, clinical profiles of the study participants, the drug-resistance pattern to first-line and second-line anti-TB drugs, and the frequency of associated mutations. Association of drug resistance of *M. tuberculosis* with geographic location, demographic data, and clinical data was assessed. Bi-variable and multi-variable logistic regression were employed to assess the association. Statistical significance was considered at a P-value of less than 0.05. Finally, the results were presented using tables and figures.

4.10. Ethical considerations

The laboratory analysis was performed on stored culture-positive isolates. Ethical clearance was obtained from the Departmental Research Ethics Review Committee (DRERC) of the Department of Microbiology, Immunology and Parasitology, College of Health Sciences, School of medicine, Addis Ababa University. Ethical approval for the amendment of the original protocol was obtained from the Ethiopian Public Health Institute Institutional Review Board (EPHI-IRB) before the execution of the study. All participants' information and results were kept confidential; the participants were not identified by their names; instead, unique study identification number was used for analysis.

4.11. Dissemination of Results

The findings of this study will be submitted and publicly presented to Addis Ababa University, Department of Microbiology, Immunology and Parasitology forpartial fulfilment of MSc in

Medical Microbiology. Also, a technical report will be submitted to Ethiopian Public Health Institute. The findings will also be presented to the medical and scientific community through different seminars and symposia. Moreover, the findings of this study will be published in peer-reviewed scientific journals.

5. Results

5.1. Demographic and clinical characteristics of study participants

In this study, a total of 127 mycobacterial isolates that were recovered during subculturing were utilized for data analysis. Seventy-nine (61.4%) of the isolates were from male participants, and 1(0.8%) isolate was from health care worker participant. The mean age of the study participants was 31 years (SD±12.29), that ranges from 9 to 71 years. Forty-six (36.2%) of the isolates were from participants in the age-group of 25-34 years. The majority (99, 78.0%) of the isolates were from the Amhara region. Ninety-nine (78.0%) of the isolates were from new cases. The majority (120, 94.5%) of the participants were presented with more than one sign and symptoms, and 91(70.9%) had indicative chest X-ray finding for TB. The predominant symptoms were chronic cough for more than 2 weeks (126, 99.2%), weight loss (121, 95.3%), and fever (115, 90.6%). Twenty-nine (22.8%) had previous contact history with TB patients. Twenty-three (18.1%) participants were *HIV* positive while one participant was co-infected with diabetes melitus (Table 1).

Table 1: Demographics and clinical characteristics of culture-positive participants (N=127) from Amhara, Gambella and Benishangul Gumuz regions of Ethiopia, April 2017 to December 2018.

Variables	Frequency (n)	Percentage (%)
Region		
Amhara	99	78.0
Gambella	14	11.0
Benshangul Gumuz	14	11.0
Age-group		
<15 years	4	3.1
15-24 years	39	30.7
25-34 years	46	36.2
35-44 years	17	13.4
45-54 years	10	7.9
≥55 years	10	7.9
Sex		
Male	78	61.4
Female	49	38.6
Sign and symptoms of TB		
Fever	115	90.6
Chronic cough more than 2 weeks	126	99.2
Night sweating	106	83.5
Weight loss	121	95.3
Indicative chest X-ray finding	91	70.9
Contact history with known TB patient		
Yes	29	22.8
No	98	76.6
Contact history with known MDR-TB patient		
Yes	-	-
No	127	100
Previous history of TB/MDR TB		
New case	99	78.0
Relapse case	17	13.4
Treatment after failure	5	3.9
Treatment after loss to follow-up	6	4.7
Diabetes Mellitus (DM)		
Yes	1	0.8
No	126	99.2
***HIV* status**		
Positive	23	18.1
Negative	104	81.9
Health care worker		
Yes	1	0.8
No	126	99.2

5.2. Anti-tuberculosis drug susceptibility pattern of *Mycobacterium tuberculosis* Complex isolates

5.2.1. Phenotypic first-line anti-TB drug susceptibility pattern of MTBC isolates

Out of the 128 isolates, DST was not performed for one (0.8%) isolate because it was not recovered on culture. Thus, phenotypic DST for first-line anti-TB drugs was performed for 127(99.2%) isolates. One hundred (78.7%) isolates were found to be fully susceptible to the four first-line anti-TB drugs (STR, INH, RIF and EMB). Any drug resistance was detected in 27(21.3%) isolates. Resistance to STR, INH, RIF, and EMB were detected in 20 (15.7%), 25 (19.7%), 17 (13.4%), and 11 (8.7%) isolates, respectively. Mono-resistance was detected for INH (2, 1.6%) and STR (2, 1.6%) while no mono-resistance was observed for RIF and EMB. Multidrug resistance was detected in 17 (13.4%) isolates while poly-resistance was detected in 20 (15.7%) isolates. The combination of poly-resistance to first-line anti-TB drugs was; (18, 14.2%) for (STR+INH), (13, 10.2%) for (STR+RIF), (9, 7.1%) for (STR+EMB), (11, 8.7%) for (INH+EMB), (9, 7.1%) for (RIF+EMB), (9, 7.1%) for (STR+INH+EMB) and (8, 6.3%) for (STR+RIF+EMB). Among all isolates, eight (6.3%) isolates were resistant to all of the four first line anti-TB drugs (STR, INH, RIF and EMB) (Table 2).

Table 2: Phenotypic drug resistance pattern of *M. tuberculosis* isolates (n=127) for first-line anti-TB drugs in Amhara, Gambella and Benishangul Gumuz regions of Ethiopia.

The pattern of drug resistance	New cases, n (%)	Retreatment cases, n (%)	Total cases, n (%)
Resistance			
Any drug-resistance	8(6.3)	19(15.0)	27(21.3)
Susceptible (4 tested dugs)	89(70.1)	11(8.7)	100(78.7)
Resistance to individual drugs			
STR	6(4.7)	14(11.0)	20(15.7)
INH	6(4.7)	19(15.0)	25(19.7)
RIF	2(1.6)	15(11.9)	17(13.4)
EMB	1(0.8)	10(7.9)	11(8.7)
Mono-resistance			
Mono STR	2(1.6)	-	2(1.6)
Mono INH	2(1.6)	-	2(1.6)
Mono RIF	-	-	-
Mono EMB	-	-	-
Two drug resistance			
STR + INH	4(3.1)	14(11.0)	18(14.2)
STR + RIF	2(1.6)	11(8.7)	13(10.2)
STR + EMB	1(0.8)	8(6.3)	9(7.1)
INH + RIF	2(1.6)	15(11.8)	17(13.4)
INH + EMB	1(0.8)	10(7.9)	11(8.7)
RIF + EMB	1(0.8)	8(6.3)	9(7.1)
MDR	2(1.6)	15(11.8)	17(13.4)
Three drug resistance			
STR + INH + RIF	2(1.6)	11(8.7)	13(10.2)
STR + INH + EMB	1(0.8)	8(6.3)	9(7.1)
STR + RIF + EMB	1(0.8)	7(5.5)	8(6.3)
INH + RIF + EMB	1(0.8)	8(6.3)	9(7.1)
STR + INH + RIF + EMB	1(0.8)	7(5.5)	8(6.3)

EMB: Ethambutol; INH: Isoniazid; RIF: Rifampicin; STR: Streptomycin; "-": Not identified

5.2.2. Genotypic first-line anti-TB drug susceptibility pattern of MTBC isolates

Analysis of the RIF and INH resistance profile of 127 isolates using the GenoType®MTBDRplus test showed that 101 (79.5%) isolates were sensitive to both INH and RIF drugs. The genotypic DST showed one additional sensitive case from that of the phenotypic DST. Isoniazid resistance was detected in 26 (20.5%) isolates, which included 8 (6.3%) isolates which were INH mono-resistant. RIF resistance was found in 19 (15.0%) isolates. All these 19 RIF resistant isolates were also resistant to INH and were therefore MDR-TB (Figure 2).

***Figure 2*:** Genotypic drug susceptibility pattern of *M. tuberculosis* isolates (n=127) for first-line anti-TB drugs using GenoType MTBDRplus VER2 in Amhara, Gambella and Benshangul Gumuz regions of Ethiopia

5.2.3. Drug susceptibility testing for second-line anti-TB drugs and pyrazinamide

Phenotypic second-line anti-TB drugs and pyrazinamide resistance pattern of multidrug resistant MTBC isolates

Phenotypic second-line anti-TB drugs and pyrazinamide (PZA) susceptibility testing was performed for all the MDR-TB isolates (n=19). Of all MDR isolates tested, 13(68.4%) isolates were found to be fully susceptible to all nine second-line anti-TB tested drugs (Am, Cm, Cfx, Eto, Km, Lvx, Lz, Mfx and Ofx). Ethionamide resistance was observed in 5(26.3%) of isolates, which included 4(21.1%) of isolates with Eto mono-resistance. Kanamycin resistance was detected in 1(5.3%) isolate, which was also Km mono-resistance. Resistance to Ofx was found in 1(5.3%) isolate. This isolate was also resistant to Cfx, Lvx, Lz. and Mfx. Pre-XDR TB was observed in 2(10.5%) MDR isolates. Besides, 16(84.2%) isolates became resistant to PZA (Table 3).

Table 3: Phenotypic drug-resistance pattern of multidrug resistant *M. tuberculosis* Complex isolates for second-line anti-TB drugs and PZA in Amhara, Gambella and Benshangul Gumuz regions of Ethiopia (n=19).

Second-line drug-resistance pattern	New cases, n (%)	Retreatment cases, n (%)	Total cases, n (%)
Susceptible to tested second line drugs	2(10.5)	11(57.9)	13(68.4)
Susceptible to PZA	1(5.3)	2(10.5)	3 (15.8)
Any Second-line anti-TB drugs resistance			
Am	-	-	-
Cm	-	-	-
Eto	-	5(26.3)	5(26.3)
Km	-	1(5.3)	1(5.3)
Ofx	-	1(5.3)	1(5.3)
Cfx	-	1(5.3)	1(5.3)
Lvx	-	1(5.3)	1(5.3)
Lz	-	1(5.3)	1(5.3)
Mfx	-	1(5.3)	1(5.3)
PZA	1(5.3)	15(78.9)	16(84.2)
Mono drug-resistance	-	4(21.1)	4(21.1)
Mono Eto	-	1(5.3)	1(5.3)
Mono Km			
Two or more Second-line anti-TB drugs resistance			
Eto + Ofx + Cfx + Lvx + Lz + Mfx	-	1(5.3)	1(5.3)
Pre-XDR	-	2(10.5)	2(10.5)

Am: Amikacin; Cm: Capreomycin; Cfx: Clofazamin; Eto: Ethionamide; Km: Kanamycin; Lvx: Levofloxacin; Lz: Linezolid; Mfx: Moxifloxacin; Ofx: Ofloxacin; PZA: Pyrazinamide; "-": Not identified

Genotypic second-line anti-tuberculosis drugs resistance pattern of multidrug resistant MTBC isolates

Analysis of the 19 MDR isolates for fluoroquinolones and second-line injectable anti-TB drugs using the GenoType®MTBDRsl assay revealed that 17(89.5%) isolates were sensitive to both fluoroquinolones and second-line injectable anti-TB drugs while two(10.5%) isolates from previously treated TB patients were resistant to fluoroquinolones, resulting in Pre-XDR-TB. However, no resistance to any of the second-line injectable drugs was detected (Figure 3).

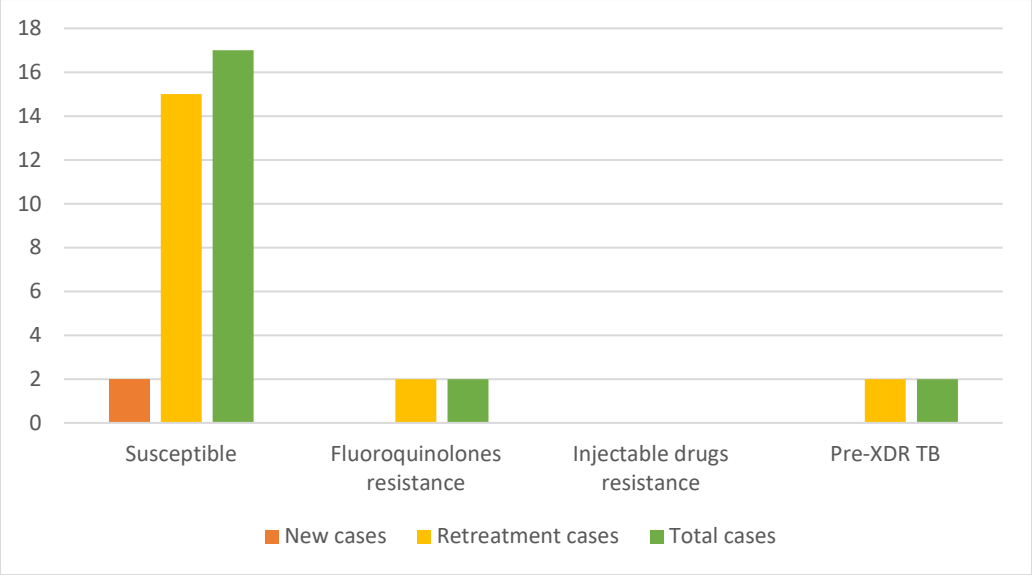

***Figure 3*:** Genotypic drug-resistance pattern of multidrug resistant *M. tuberculosis* isolates for second-line anti-TB drugs using GenoType MTBDRsl in Amhara, Gambella and Benshangul Gumuz regions of Ethiopia (n=19)

Mutations associated with resistance to first-line anti-TB drugs; rifampicin and isoniazid

Among the 26 INH-resistant isolates, 20(76.9%) had mutations in the *katG MUT1* gene with missing wild type in *katG* (amino acid change of S315T1), 2(7.7%) had mutations in the *katG MUT2* gene with missing wild type in *katG* (amino acid change of S315T2), 1(3.8%) had *katG WT* missing, and 3(11.5%) had mutations in *inhA* promoter region with missing wild type in *inhA* gene (amino acid change of C15T). Of the 19 RIF-resistant isolates, 10(52.6%) had mutations in *rpoB MUT3* with missing wild type in *rpoB WT8(*amino acid change of S531L), 3(15.8%) had mutations in *rpoB MUT2A* with missing wild type in *rpoB WT7* (amino acid change of H526Y), 2(10.5%) had mutations in *rpoB MUT1* with missing wild type both in *rpoB WT3* and *rpoB WT4* (an amino acid change of D516V), 2(10.5%) had no mutations in *rpoB* gene while missing wild type in *rpoB WT7*, 1(5.3%) had no mutations in *rpoB* gene while missing wild type both in *rpoB WT3* and *rpoB WT4*, and 1(5.3%) had mutations in *rpoB MUT3* while no missing wild type in *rpoB* gene (Table 4).

Table 4: Frequency and pattern of mutations for isoniazid and rifampicin-resistant *M. tuberculosis* isolates identified using GenoType MTBDRplus VER2 assay.

Anti-TB drugs	No. of resistant isolates	The pattern of gene mutations wild type/mutant	Amino acid change	Frequency (%)
Isoniazid	26	Δ*katG WT/ katG MUT1*	S315T1	20(76.9)
		Δ*katG WT/ katG MUT2*	S315T2	2(7.7)
		Δ*katG WT/* ND	-	1(3.8)
		Δ*inhA WT/ inhA MUT1*	C15T	3(11.5)
Rifampicin	19	Δ*rpoB WT8/rpoB MUT3*	S531L	10(52.6)
		Δ*rpoB WT7/rpoB MUT2A*	H526Y	3(15.8)
		Δ*rpoB WT3 and rpoBWT4*/*rpoB MUT1*	D516V	2(10.5)
		Δ*rpoB WT7*/ND	-	2(10.5)
		Δ*rpoB WT3 and rpoBWT4*/ ND	-	1(5.3)
		No missing *rpoB WT*/*rpoBMUT3*	-	1(5.3)

Δ, deletion. MUT, mutant, ND, no mutation detected at the mutant probe,"-": Not identified

Mutations associated with resistance to second-line anti-TB drugs; fluoroquinolones and injectables

Out of the two second-line anti-TB resistant isolates, 1(5.3%) had mutation in the *gyrA MUT* gene and missing wild type in *gyrA* with no known amino acid change, and 1(5.3%) had no mutant gene while missing wild type in *gyrA* gene, with an amino acid change of G88A/G88C (Table 5).

Table 5: Frequency and pattern of mutations identified for second-line anti-TB drug-resistant *M. tuberculosis* isolates using GenoType MTBDRsl VER2

Anti-TB drugs	No. of resistant isolates	The pattern of gene mutations wild type/mutant	Amino acid change	Frequency (%)
Fluoroquinolones	2	Δ*gyrA WT/ gyrA MUT*	-	1(5.3)
		Δ*gyrA WT/* ND	G88A/G88C	1(5.3)

Δ, deletion. MUT, mutant, ND, no mutation detected at the mutant probe, r, resistant.

5.3. Spoligotyping (Spacer-Oligotyping)

Spoligotyping was performed for all of the 127 culture recovered clinical isolates. Among those, 119 isolates had interpretable spoligotyping results and therefore were included in the final analysis of our study. The remaining nine isolates were removed from the final analysis due to uninterpretable spoligotyping results. The 119 isolates generated 43 different spoligotyping patterns using the SITVIT2 and MIRU-VNTRplus databases. Eighty-seven (73.1%) isolates generated 26 distinct spoligotype patterns. While the remaining 31(26.0%) isolates had 17 different spoligotype patterns that were not reported by the SITVIT2 and MIRU-VNTRplus databases. Out of the 26 distinct spoligotype patterns, 16 patterns represented the corresponding clusters containing 2 to 19 isolates per each cluster. The remaining 10 patterns represented a single isolate (non-clustered spoligotype pattern) in the database. Out of the 17 unique patterns, four patterns represented clustered isolates each containing 2 to 8 isolates while the rest 13 patterns represented a single isolate. Of the shared types identified, the predominant strain was SIT149 of the T3-ETH subfamily containing 19(15.9%) isolates and followed by SIT289 of CAS1-Delhi subfamily with 13(10.9%) isolates. The third and fourth most common strains identified were SIT53 of T1 subfamily and SIT584 of T2 subfamily, consisting 8(6.7%) and 7(5.9%) isolates, respectively.

In regards to the family assignment, we found four major families in our study. These are the T family 54/119(45.4%), CAS family 33/119(27.7%), AFRI family 12/118(10.1%) and Haarlem family 10/119(8.4%). Tuscany (T) family consisted T3-ETH 22/119(18.5%), T 14/119(11.8%), T2 8/119(6.7%), T3 6/119(5.0%), and T1-RUS2 4/119(3.4%) sublineages whereas the CAS family consist of CAS 5/119(4.2%), CAS1-Delhi 24/119(20.2%), CAS1-Kili 4/119(3.4%) sublineages. The Haarlem family also consisted H1 2/119(1.7%), H2 1/119(0.8%), H3 6/119(5.0%), H3-Ural-1 1/119(0.8%) sublineages. Other families represented were X1 family 6/119(5.0%); LAM family 2/119(1.7%): LAM3 1/119(0.8%) and LAM9 1/119(0.8%) and Manu family 2/119(1.7%): Manu1 1/119(0.8%), Manu2 1/119(0.8%). In this study, classification of the isolates by Major Lineage showed Euro-American lineages 72/119(60.5%), East-African-Indian lineages 35/119(29.4%) and Mycobacterium-africa 12/119(10.1%) (Table 6).

Table 6: Spoligotype patterns of *M. tuberculosis* isolates (n=119) obtained from presumptive TB/MDR-TB patients of Amhara, Gambella, and Benishangul-Gumuz of Ethiopia.

S. No	SIT	Spoligotype pattern	Spoligotype43 (Octal code)	Major Lineage by CBN	Clade by KBBN	Lineage by SITVIT2	Number of isolates (%)
1.	1074		777777607760761	EA	LAM	LAM9	1(0.8)
2.	119		777776777760771	EA	X1	X1	3(2.5)
3.	1200		703777747777771	EAI	Manu1	Unknown	1(0.8)
4.	134		777777777720631	EA	H3	H3	2(1.7)
5.	1475		777357777760771	EA	T	T1	1(0.8)
6.	149		777000377760771	EA	T3-ETH	T3-ETH	19(16.0)
7.	1745		773737777760771	EA	T3	T3	2(1.7)
8.	21		703377400001771	EAI	CAS1-Kili	CAS1-Kili	3(2.5)
9.	22		703777400001771	EAI	CAS	CAS1-Delhi	3(2.5)
10.	2298		777777647760771	EA	T	T1	2(1.7)
11.	26		703777740003771	EAI	CAS1-Delhi	CAS1-Delhi	3(2.5)
12.	289		703777740003571	EAI	CAS1-Delhi	CAS1-Delhi	13(10.9)
13.	302		777756777760771	EA	X1	X1	2(1.7)
14.	3047		777357777720671	EA	H3	H3	1(0.8)
15.	3134		777737377720771	EA	H3	H3	2(1.7)
16.	3141		177000377760771	EA	T3-ETH	T3-ETH	1(0.8)
17.	3183		000000077760771	EA	T1-RUS2	Unknown	2(1.7)
18.	336		777776777760731	EA	X1	X1	1(0.8)
19.	35		777737777420771	EA	H3-Ural-1	Ural-1	1(0.8)
20.	37		777737777760771	EA	T3	T3	4(3.4)
21.	46		777777770000000	EA	H1	Unknown	2(1.7)
22.	52		777777777760731	EA	T2	T2	1(0.8)
23.	53		777777777760771	EA	T	T1	8(6.7)
24.	584		777775777760731	EA	T2	T2	7(5.9)
25.	764		777757777720771	EA	H3	H3	1(0.8)
26.	794		703757740003771	EAI	CAS1-Delhi	CAS1-Delhi	1(0.8)

Table 6: … cont'd

S. No	SIT	Spoligotype pattern	Spoligotype43 (Octal code)	Major Lineage by CBN	Clade by KBBN	Lineage by SITVIT2	Number of isolates (%)
27.	Orphan		000000047720771	EA	T1-RUS2	Unknown	1(0.8)
			000000047760771	EA	T1-RUS2	Unknown	1(0.8)
			477777777605671	Unknown	Unknown	Unknown	1(0.8)
			603777700000771	EAI	CAS	Unknown	1(0.8)
			700000044037771	EAI	H2	Unknown	1(0.8)
			700000044177611	*M. africa*	AFRI	Unknown	1(0.8)
			700000044177771	*EAI*	CAS	Unknown	4(3.4)
			700000047177771	*M. africa*	AFRI	Unknown	8(6.7)
			703757740003571	EAI	CAS1-Delhi	Unknown	4(3.4)
			703773740003571	EAI	CAS1-Delhi	Unknown	1(0.8)
			703777700001171	EAI	CAS1-Delhi	Unknown	1(0.8)
			703777740003531	EAI	CAS1-Delhi	Unknown	1(0.8)
			776157647760771	EA	LAM3	Unknown	1(0.8)
			776157647762771	EA	Manu2	Unknown	1(0.8)
			777200377760771	EA	T3-ETH	Unknown	2(1.7)
			777355777760731	EA	T2	Unknown	1(0.8)
			777777474760771	EA	T	T-H37Rv	1(0.8)

CBN: a conformal Bayesian network; EA: Euro American; IO: Indo-Oceanic; EAI: East African-Indian; KBBN: knowledge-based Bayesian network

5.4. Associated factors of drug-resistant tuberculosis

In the present study, statistically significant association between the study variables and any type of anti-TB drug resistance was not found for region (X^2=1.74; p=0.410), sex (X^2=0.125; p=0.724), age group (X^2=3.471; p=0.628) and type of lineage (X^2=4.548; p=0.337). While, statistically significant association with any type of anti-TB drug resistance was found for previous TB history (X^2=46.59; p<0.001) and previous TB treatment outcome (X^2=47.677; p<0.001) (table 7).

Table 7: Association of study variables with drug resistance TB among MTBC isolates collected from Amhara, Gambella and Benshangul Gumuz regions of Ethiopia (n=127).

Variables	Total N (%)	Drug resistance of *M. tuberculosis* N (%)		X^2	p-value
		No	Yes		
Regions					
Amhara	99 (78.0)	75 (59.0)	24 (18.89)		
Gambella	14 (11.0)	13 (10.2)	1 (0.78)	1.784	0.410
Benshangul-Gumuz	14 (11.0)	11 (8.66)	3 (2.36)		
Sex					
Male	78 (61.41)	60 (47.24)	18 (14.17)	0.125	0.724
Female	49 (38.58)	39 (30.70)	10 (7.87)		
Age-group (years)					
5-14	4 (3.14)	3 (2.36)	1 (0.78)		
15-24	39 (30.70)	34 (26.77)	5 (3.93)		
25-34	46 (36.22)	33 (25.98)	13 (10.23)	3.471	0.628
35-44	17 (13.38)	13 (10.23)	4 (3.14)		
45-54	10 (7.87)	7 (5.51)	3 (2.36)		
≥55	12 (7.08)	9 (4.72)	2 (1.57)		
Previous TB history					
Yes	27 (21.26)	8 (29.6)	19 (70.4)	46.59	<0.001
No	100 (78.7)	91 (91.0)	28 (22.0)		
Previous TB treatment outcome					
New case	99 (77.95)	90 (70.08)	9 (7.08)		
Relapse case	17 (13.38)	7 (5.51)	10 (7.87)	47.677	<0.001
Treatment after failure	5 (3.93)	-	5 (3.93)		
Treatment after loss to follow-up	6 (4.72)	2 (1.57)	4 (3.14)		
Major Lineage					
Euro-American	72 (60.5)	55 (46.2)	17 (14.3)		
East-African-Indian	35 (29.4)	25 (21.0)	10 (8.4)	2.035	0.362
Mycobacterium-africa	12 (10.1)	11 (9.2)	1 (0.8)		
Not valid	8 (100)	8 (100)	-		

"-": Not detected

6. Discussions

In this study, we investigated the molecular epidemiology and drug resistance pattern of *M. tuberculosis* isolates circulating in the Amhara, Gambella and Benishangul-Gumuz regions of Ethiopia. Furthermore, the study investigated mutation patterns of *M. tuberculosis* isolates and the association of drug resistance of *M. tuberculosis* with geographic location, age, sex, previous TB history, previous TB treatment outcome and *M. tuberculosis* lineages. The study identified a high proportion of drug-resistant mycobacterial isolates. The study also found highest monoresistance to be against isoniazid and streptomycin. In addition, the study indicated the presence of a significant proportion of MDR and Pre-XDR TB isolates in these regions. Moreover, INH and RIF drug resistance showed highest mutation in the *katG MUT1* gene (S315T1) and *rpoB* gene (S531L) respectively. Also, the FLQ resistance were associated with the mutations in *gyrA* gene. Besides, the study revealed that the SIT149 was the highly predominant strain. Furthermore, diversified mycobacterial lineages were observed in this study, but the Euro American lineage was predominant.

Drug resistance pattern of *M. tuberculosis*

This study revealed that 21.3% of the *M. tuberculosis* isolates were resistant to any of the first-line anti-TB drugs using phenotypic DST. Similarly, other studies conducted in Ethiopia reported relatively comparable results of 20.2%, 24.8% and 23.3% (Adane *et al*., 2015; Ali *et al*., 2016; Tilahun *et al*., 2018). However, a higher resistance proportion was reported from Southwest Ethiopia (58.6%), Addis Ababa (54.4%) and Northwest Ethiopia (34.1%) (Abdella *et al*., 2015; Mesfin *et al*., 2018; Yigzaw *et al*., 2021). On the contrary, a lower resistance proportion was reported in southern Ethiopia (11.1%), Addis Ababa (13.3%) and Northwest Ethiopia (16.1%) (Wondale *et al*., 2018; Damena *et al*., 2019; Lobie *et al*., 2020). The differences might be due to the differences in patient characteristics. Accordingly, the study indicated the presence of drug resistance in the three regions of the country.

The highest proportion of resistance was found for INH (19.7%). Likewise, previously conducted studies in the country reported higher resistance to INH compared to other first-line drugs (Abdella *et al*., 2015; Lobie *et al*., 2020; Mesfin *et al*., 2018; Yigzaw *et al*., 2021). Specifically, up to 51.4% INH resistance was reported in southwest Ethiopia (Abdella *et al*., 2015). This high INH resistance might be due to the use of INH for several years by the country's tuberculosis control program. Hence, this could significantly affect the country's tuberculosis control program, as INH is one of

the potent drugs used for MDR-TB treatment and prophylaxis drug for contacts of TB patients, latent TB patients and HIV/AIDS patients. Therefore, the identification of high INH resistance in this and other previous Ethiopian studies could imply the need of performing full first-line phenotypic DST to all TB patients in the country so that they can be treated with the right drug. However, there are studies that reported the highest resistance to STR (14.1%) and (19.8%) from the southeast and northwest Ethiopia respectively (Haile *et al*., 2020; Yigzaw *et al*., 2021).

Mono-resistance in this study was found only for INH and STR with 1.6% equal proportion. This report is supported by a relatively comparable finding 1.1% of previous national survey (Getahun *et al*., 2015). However, higher INH mono-resistance (8.7%) was reported from northwest Ethiopia (Alelign *et al*., 2019). Additionally, most of the previous studies that were conducted in different parts of Ethiopia (Getahun *et al*., 2015; Haile *et al*., 2020; Hamusse *et al*., 2016; Tilahun *et al*., 2018; Yigzaw *et al*., 2021) reported higher INH or STR mono-resistance than other first-line anti-TB drugs, which is a similar finding to the current study. This could be linked to poor treatment outcome in previously treated tuberculosis patients and could also be because of the multi-year use of the INH and STR drugs by the country's tuberculosis control program.

The study also revealed MDR-TB prevalence of 13.4%. This finding relatively corresponds to the Ethiopian profile in the 2020 WHO global TB report (World Health Organization., 2020). However, in another report from Southwest Ethiopia, higher MDR-TB prevalence was reported (28%) (Tadesse *et al*., 2016). On the other hand, lower prevalences of MDR-TB were reported by other studies: 4.4% from a national survey, 2.4% from Benishangul-Gumuz Ethiopia, and 1.2% from central Ethiopia (Getahun *et al*., 2015; Lobie *et al*., 2020; Tilahun *et al*., 2018). However, the data from the first reports were obtained from studies that included isolates from resource- limited regions of the country, which might be a potential cause for the observed difference. In the current study, poly-drug resistance was detected in 60.6% of the isolates. Previous studies in different parts of Ethiopia also reported the presence poly-drug resistance (Getahun *et al*., 2015; Tilahun *et al*., 2018; Wondale *et al*., 2018). The presence of poly-drug resistance could negatively affect patient's treatment outcome and also lead to treatment failure and disease relapse. Furthermore, the use of Xpert MTB/RIF assay as an initial diagnostic test to identify TB and detect RIF resistance within the country may lead to undiagnosed poly-drug resistant TB cases.

Besides, the study identified that pre-XDR TB is also circulating in the country where it detected 2 (10.5%) pre-XDR cases, both of which being from previously treated cases. In line with this,

other previous studies from Ethiopia also reported detection of pre-XDR cases, where it was observed that previously treated cases were more likely to develop drug resistant TB compared to new cases (Bedru *et al*., 2021; Tesema *et al*., 2021; Shibabaw *et al*., 2020). The pre-XDR cases in this study occurred due to resistance to fluoroquinolones, similar to some reports from other studies in Ethiopia (Tesema *et al*., 2021; Shibabaw *et al*., 2020; Welekidan *et al*., 2020). The increasing use of fluoroquinolone drugs for the treatment of other bacterial infections including respiratory, gastrointestinal and urinary tracts infections (Mama *et al*., 2020) might contribute for the increased rate of resistance to this drug. The circulating pre-XDR strains identified in this and previous studies in Ethiopia are alarming to the national TB prevention and control program.

Mutations associated with drug resistant *M.tuberculosis*

In the present study, all the first-line and second line phenotypic DST result were concordant with the genotypic method except one isolate that was rifampicin resistance in the phenotypic DST but susceptible in the MTBDRplus. We assessed the mutations associated with drug resistance using the MTBDRplus and MTBDRsl assays. The result revealed that highest (76.9%) mutation for INH resistance was detected in the *katG MUT1* gene with missing wild type that corresponds to amino acid change of S315T1, which was also supported by previous studies (Ejo *et al*., 2021; Mekonnen *et al*., 2015; Taddesse *et al*., 2016; Kebede *et al*., 2017). The second (11.5%) most common identified mutation for INH resistance was found in the *inhA* promoter region with missing wild type in *inhA* gene that corresponds to amino acid change of C15T. Likewise, this type of mutation was reported from different parts of the country (Taddesse *et al*., 2016; Welekidan *et al*., 2020; Brhane *et al*., 2017). The third (7.7%) most abundant mutation identified was the *katG MUT2* gene with missing wild type in *katG* which corresponds to amino acid change of S315T2. This mutation was also reported previously by few Ethiopian studies (Mekonnen *et al*., 2015; Wondale *et al*., 2018). This study also revealed that 3.8% of INH resistant isolates developed resistance with unknown mutation (only wild type in *katG* missed). Likewise, INH resistance without identified mutation was reported in previous studies conducted in Ethiopia (Abate *et al*., 2014; Welekidan *et al*., 2020). The unknown mutations might be due to mutations outside the *inhA* and *katG* regions.

The current study identified highest mutations in *rpoB MUT3* (52.6%) with missing wild type in *rpoB WT8* (amino acid change of S531L). This result is supported by different studies conducted so far in Ethiopia (Brhane *et al*., 2017; Kebede *et al*., 2017; Damena *et al*., 2019). Additionally, the study revealed mutations in *rpoB MUT2A* with missing wild type in *rpoB WT7* (amino acid

change of H526Y) among 15.8% of the isolates tested. Likewise, mutation in this region was previously reported in Etiopia (Abate *et al*., 2014; Brhane *et al*., 2017; Kebede *et al*., 2017; Welekidan *et al*., 2020). Furthermore, the study found that 10.5% of the isolates lacked mutations while wild type in *rpoB WT7* was missing. Additionally, 5.3% of the isolates RIF resistant isolates developed resistance with no mutations whereas the wild types *rpoB WT3* and *rpoB WT4* were missing. The cases of no mutation while the wild type is missing in *rpoB* gene can be because of mutations outside the 81bp regions of the *rpoB* gene. In fact, such observations were not new in the country as previous studies reported supportive findings (Mekonnen *et al*., 2015; Gashaw *et al*., 2021; Tilahun *et al*., 2020; Welekidan *et al*., 2021).

Among MDR-TB isolates tested with GenoType®MTBDRsl assay, about 89.5% were sensitive to the second-line anti-TB drugs. This finding is supported by other Ethiopian studies that were conducted in different parts of the country (Damena *et al*., 2019; Welekidan *et al*. 2021). The study detected mutations in 2 (10.5%) MDR-TB isolates that were directed against fluoroquinolones, as was reported from previous other studies in the country (Damena *et al*., 2019; Shibabaw *et al*. 2020; Welekidan *et al*. 2021). One of these two isolates had a mutation in the *gyrA MUT* gene with a missed wild type in *gyrA* gene while the other one had a missing wild type in *gyrA* gene but no mutant. According to current WHO definitions, these fluoroquinolone resistant isolates fall into Pre-XDR-TB classification. Similarly, recent studies conducted in Ethiopia reported that fluoroquinolone resistance was associated with *gyrA* gene (Damena *et al*., 2019; Shibabaw *et al*. 2020; Welekidan *et al*. 2021). Taken together, these findings suggest the need for establishing and expanding laboratories that can perform genotypic assays to diagnose TB and detect drug resistance as early as possible, especially in resource limited regions of the country, so that the MDR-TB patients can get the drug resistance results for the first-and second-line anti-TB drugs resistance results timely. Besides, the genotypic method could help to understand the molecular epidemiology of MTBC in the absence of sequencing.

Molecular epidemiology of *M. tuberculosis*

In regards to the genetic diversity of mycobacteria isolates in this study, as determined by using Spoligotyping, all the isolates that have interpretable Spoligotyping results were *M. tuberculosis*. The majority (73.7%) of these clinical isolates showed 26 distinct Spoligotyping patterns which were previously reported in the SITVIT2 and MIRU-VNTRplus databases. Other previously conducted studies from Northwest, Eastern Amhara and Southern Omo in Ethiopia supported this

finding (Ejo *et al*., 2021; Esmael *et al*., 2014; Wondale *et al*., 2020). However, a good number of the remaining isolates (26.3%) which showed 17 different spoligotype patterns that were not previously reported in the databases. This finding is also supported by other similar Ethiopian studies, where new Spoligitype patterns that are not found in the SITVIT2 and MIRU-VNTRplus databases were reported (Ejo *et al*., 2021; Esmael *et al*., 2014; Wondale *et al*., 2020). Detection of high proportion of mycobacterial isolates that were not previously reported in the databases in this study could be either because of low number of previous studies in the study areas or due to unknown mycobacterial strains are found in the study area.

The study classified the majority of the *M. tuberculosis* isolates (92.4%) into four families, among which the T family (45.8%) was the predominant one followed by the CAS family (28.0%). The finding that these two families predominate in Ethiopia was also reported previously by other investigators (Damena *et al*., 2019; Ejo *et al*., 2021; Esmael *et al*., 2014; Wondale *et al*., 2020). The remaining two dominant families were the AFRI (10.2%) and Haarlem (8.5%) families. Other previously conducted Ethiopian studies also supported this finding (Esmael *et al*., 2014; Yimer *et al*., 2015).

The study further identified different lineages, among which the Euro American (60.5%) was predominant. Other previous studies from Ethiopia also reported that this lineage was predominant (Abebe *et al*., 2019; Alelign *et al*., 2019; Gulich *et al*., 2015; Nuru *et al*., 2015; Tessema *et al*., 2013; Firdessa *et al*., 2013; Damena *et al*., 2019; Disassa *et al*., 2015; Wondale *et al*., 2020). The East-African-Indian lineage (29.4%) was the second dominant lineage in this study which is concordant with other Ethiopian studies (Abebe *et al*., 2019; Disassa *et al*., 2015; Gulich *et al*., 2015; Nuru *et al*., 2015; Damena *et al*., 2019; Disassa *et al*., 2015). Furthermore, the study identified the Mycobacterium-africa lineage (10.1%) which mainly specific to the West and Central African regions. This finding was also supported by other Ethiopian study (Abebe *et al*., 2019) and could further suggest the transmission of the disease from West and Central African counties.

Interms of sublineages, the study detected several sublineages, among which the CAS1-Delhi (20.2%) was the predominant one which is a comparable finding with the other Ethiopian findings (Ejo *et al*., 2021; Wondale *et al*., 2020). The second dominant sublineage in this study was T3-ETH (18.5%). This sublineage was also commonly reported by other similar Ethiopian studies (Ejo *et al*., 2021; Esmael *et al*., 2014; Wondale *et al*., 2020). With the detection of the CAS1-Delhi

and T3-ETH which are strongly associated with drug-resistant TB (Deribew *et al*., 2012), the country should focus on detecting and treating drug-resistant TB as soon as possible to halt the transmission of the disease in the community.

Regarding the strain types of this study, the SIT149 (15.9%) was the predominant one followed by the SIT289 (10.9%) which is a comparable finding with the other reports from Ethiopia (Damena *et al*., 2019; Lobie *et al*., 2020; Tilahun *et al*., 2018). The detection of similar spoligotyping patterns of SIT149 (19 isolates) and SIT289 (13 isolates) could indicate an active transmission of tuberculosis within the study areas.

Association of drug resistant *M. tuberculosis* with different factors

This study also tried to assess the factors associated with drug resistance TB using the available study variables. The findings revealed that previous history of TB treatment had a statistical significant association with drug resistant TB. This might be due to poor treatment adherence to first-line anti-TB drugs. Likewise, other previous studies reported higher risk of TB in previously treated patients (Abdella *et al*., 2015; Adane *et al*., 2015; Alemu *et al*., 2021; Alene *et al*., 2019; Mekonnen *et al*., 2015; Wondale *et al*., 2018). Besides, the study revealed that previous TB treatment outcome is strongly associated with drug resistant TB, where other previously conducted Ethiopian studies also supported this finding (Abdella *et al*., 2015; Alemu *et al*., 2021).

7. Strength and Limitation of the Study

7.1. Strength of the study

The study provides additional information on the molecular epidemiology of *Mycobacterium tuberculosis* in the three regions. Although limited in number of study participants, the report on the two resource-limited regions of the country, Gambella and Benishangul Gumuz, might add some more upon the already available data of the tuberculosis control program of the country. The other strength could be that second-line drug susceptibility testing included the newly endorsed and repurposed drugs such as Linezolid and Clofazimine for MDR-TB isolates.

7.2. Limitation of the study

Finally, the findings from this study should be interpreted by considering the following limitations. First, in the present study, MIRU-VNTR (Mycobacterial interspersed repetitive unit variable number tandem repeat) and sequencing were not conducted. Second, the study was based on stored isolates collected for other purpose which hindered assessment of other study variables that might be associated with drug resistant TB. Besides, the isolates were not evenly distributed within the three regions indicated.

8. Conclusions and Recommendations

8.1. Conclusion

In this study, significant proportion of drug resistant TB (monoresistance, MDR, Pre-XDR) was detected which could imply the drug resistant TB is a major public health problem in the country. Consequently, the country should focuse on the strategies that help for early detection and treatment of drug-resistant TB. The most frequent *rpoB* gene mutation was observed at the *S531L* gene, while, the most frequent mutation among isoniazid-resistant cases occurred at the *katG S315T1* gene region. The two Pre-XDR TB isolates were associated with the *gyrA* gene mutaions. Furthermore, the SIT149 (16.1%) and SIT289 (11.0%) were the two predominant strains. The 17 different spoligotype patterns in 32(26.9%) isolates were not previously reported in the SITVIT2 and MIRU-VNTRplus databases, which indicates the high proportion of orphan isolates and/or the presence of new mycobacterial strains in the study area. The most frequently identified family from this study was the T family. Although a diversified mycobaterium lineages were observed in this study, the Euro American lineage and East-African-Indian lineages were the predominant lineages with CAS1-Delhi and T3-ETH sublineages which are strongly associated with the drug-resistant TB. Therefore, the country should focus to halt the transmission of drug-resistant TB.

8.2. Recommendations

We recommend further molecular characterization of the unknown mycobacterial isolates, particularly using sequencing platforms. We also recommend the phenotypic drug susceptibility testing to first- and second-line drugs including the newly endorsed and repurposed drugs like Bedaquiline and Delamanid. Besides, the country's tuberculosis control program should monitor patient adherence to ensure the patients complete the full course of treatment.

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10.Annexes

Annexe 10.1: Laboratory test protocols

10.1.1. Mycobacterial Sub-culturing

A pure, well characterized culture of *M.tuberculosis* bacteria in the active growth phase is required to perform both molecular and phenotypic identification of tuberculosis. This can be done by sub-culturing the frozen suspension of the MTBC isolates on MGIT tube as:

1. Thaw the frozen MTBC isolate suspension.
2. Vortex cryovial with MTBC isolate suspension well to mix thoroughly. Leave 10-15 minutes to allow any large clumps to settle.
3. Aseptically add 0.8 ml growth supplement without PANTA into a new MGIT tube.
4. Use inoculum from the supernatant broth of well mixed MTBC isolate suspension and make a 1:100 dilution of the thawed MTBC isolate into sterile saline.
5. Mix tube well by inverting gently several times.
6. Inoculate 0.5 ml of the 1:100 diluted specimen into the new MGIT tube.
7. Cap tube tightly and mix well by inverting gently several times.
8. Enter tube into MGIT 960 instrument, incubate at 37 ^{o}C and monitor until it becomes positive.
9. Use new tube from one to five days of positivity to set DST.

10.1.2. Phenotypic drug-susceptibility testing

Principle

Susceptibility testing in the MGIT 960 system is based on the same principles as isolation of mycobacterium from sputum (detection of growth). DST is performed using an AST (antibiotic susceptibility testing) set or TB exit, which consists of a Growth Control tube and one tube for each drug. A known concentration of drug is added to a MGIT tube, along with the specimen, and growth is compared with a drug-free control of the same specimen. If the drug is active against the mycobacterial isolate (isolate susceptible), growth will be inhibited and fluorescence will be suppressed in the drug-containing tube; meanwhile, the drug-free control will grow and show increasing fluorescence. If the isolate is resistant, growth and its corresponding increase in fluorescence will be evident in both the drug-containing and the drug-free tube. The MGIT 960 system monitors these growth patterns and can automatically interprets results as susceptible or

resistant. An isolate is defined as resistant if 1% or more of the test population grows in the presence of the critical concentration of the drug.

Procedure

Working drug solution preparation for Lyophilized SIRE drugs and second line drugs

1. Using a sterile pipette or transfer pipette, reconstitute each of the SIRE (Streptomycin, Isoniazid, Rifampicin and Ethambutol) lyophilized drug vials with 4 ml of sterile distilled water.
2. Using a sterile pipette or transfer pipette, reconstitute each of the second line TB drugs as:

Drug	Solvent	Diluent	Concentration in working solution µg/ml	Volume added to MGIT tube (µl)	Final concentration in MGIT tube (µg/ml)
Amikacin	SDW	SDW	84	100	1.0
Capreomycin	SDW	SDW	210	100	2.5
Clofazamine	DMSO	DMSO	*	100	1.0
Ethionamide	DMSO	SDW	420	100	5.0
Kanamycin	SDW	SDW	210	100	2.5
Levofloxacin	0.1 N NaOH	SDW	84	100	1.0
Linezolid	SDW	SDW	84	100	1.0
Moxifloxacin	0.1 N NaOH	SDW	21	100	0.25
Ofloxacin	0.1N NaOH	SDW	168	100	2.0

*Clofazamine working solution (WS) at low concentrations are unstable. The Working solution must be prepared every time starting from stock solution.

3. Use separate pipette for each drug.
4. Aliquot in sterile cryovial tube by considering the average test done at each run.
5. Store aliquoted drugs at -20^{0}C up to 6 months or up to the date of original expiry, whichever comes first.
6. Once thawed, discard any leftover drug and do not store or refreeze.

Inoculum preparation for MGIT DST

For DST testing:

1. Positive MGIT cultures must have pure growth of *M. tuberculosis* (ZN positive, BAP negative, MPT/MTB 64 antigen test positive; to be tested for drug susceptibility.
2. DST must not be set up on the same day a MGIT tube signals positive. The day a MGIT tube is positive by the instrument is considered Day 0.

If the MGIT culture is worked up 1 or 2 days after signaling positive,

1. Vortex each seed tube for 1 minute to break up clumps.
2. Leave the tube undisturbed for about 20 minutes to allow large clumps to settle.

If the MGIT culture is used to set up DST between 3 and 5 days after signaling positive,

1. Vortex each seed tube for 1 minute to break up clumps.
2. Let the large clumps settle for 20 minutes.
3. Dilute 1 ml of the positive broth in 4 ml of sterile saline (1:5 dilutions).
4. Use this well–mixed, diluted culture for inoculation of the drug set.

If the MGIT culture has been longer than 5 days after signaling positive, subculture into a fresh MGIT tube:

1. Vortex positive MGIT broth for 1 min. Leave 20 minutes at room temperature to allow any large clumps to settle.
2. Supplement a new MGIT tube with 0.8 ml Growth Supplement without PANTA.
3. Remove inoculum from the supernatant broth and make a 1:100 dilution of the positive MGIT tube into sterile saline
4. Mix tube well by inverting gently several times.
5. Inoculate new MGIT tube with 0.5 ml of the 1:100 diluted specimen.
6. Cap tube tightly and mix well by inverting gently several times.
7. Enter tube into MGIT 960 instrument and monitor until it becomes positive.
8. Use new tube for DST within one to five days of positivity.

Preparation of 1:100 (for growth control)

1. For 1-2 days old MGIT tube, prepare the 1:100 dilution by adding 100 µl of the seed into 9.9 ml of sterile saline.

2. For 3-5 days old MGIT tube, first prepare the 1:5 dilution by adding 1 ml of the seed into 4 ml sterile saline and then the 1:100 dilution by adding 100 µl of the 1: 5 diluted seed to 9.9 ml of sterile saline.

Inoculation into DST MGIT tube set

1. Label MGIT tubes for each test isolate with isolate number, GC (Growth Control), STR (streptomycin), INH (isoniazid), RIF (rifampicin), EMB (ethambutol), Am (Amikacin), Cm (Capreomycin), Cfx (Clofazimine), Eto (Ethionamide), Km (Kanamycin), Lvx (Levofloxacin), Lz (Lenozolid), Mfx (Moxifloxacin) and Ofx (Ofloxacin) using Epicenter.
2. Place the tubes in the following sequence from left to right: GC, STR, INH, RIF, EMB, Am, Cm, Cfx, Eto, Km, Lvx, Lz, Mfx and Ofx.
3. Aseptically add 0.8 ml MGIT SIRE Supplement to each MGIT tube (including GC labelled and drug labelled MGIT tubes).
4. Aseptically pipette 100 µl of working drug solutions to the corresponding drug labelled MGIT tubes.
5. Do not add drugs to the MGIT GC tube.
6. Using a sterile transfer pipette, inoculate 0.5 ml of the 1:100 diluted isolate into the GC MGIT tube.
7. Inoculate 0.5 ml of the organism suspension from the supernatant into each drug labelled tube.
8. Immediately recap the tube tightly and mix tubes gently by inverting 3-4 times.
9. Wipe all tubes and caps with a mycobactericidal disinfectant.

Incubation

1. Enter the inoculated set of DST specimens into the BACTEC 960 instrument using the AST set entry feature.
2. Be sure that the tubes are loaded according to the order specified above and the caps are tightly closed.
3. Open the desired MGIT 960 drawer and press the “tube entry” soft key.
4. The barcode scanner will light up.
5. Scan the inoculated MGIT tube and load into the slot identified by the MGIT 960.
6. Incubate MGIT tubes at 37^0C until the instrument flags them as positive.
7. Check MGIT 960 daily for indicator lights flagging positive.

8. Positive tubes will be displayed by the indicator light changing to green at the exact location of the tube in the instrument drawer.

Notes:

- If the GC tubes become positive in less than 4 days or remain negative up to 21 days or some other conditions occur which may affect the test results, the instrument report will be as an Error ("X"). In such situations, the test needs to be repeated.
- If a laboratory routinely experiences x200 errors when performing DST in the local patient population, the 3-5 day culture can be used undiluted as a first step. Monitor this technique very closely to ensure that an excess of x400 errors is not subsequently produced.
- The turbidity should not be less than McFarland 0.5.
- The tube cap should be tightly closed.

Result Interpretation

Once the growth control (i.e. 1 % inoculum) is positive (after a minimum of 4 days and a maximum of 13 days), interpretation of results must be made manually as "**S**" (susceptible) or "**R**" (resistant) using Epicenter from MGIT 960 system.

- An isolate is defined as "**Resistant**": when the growth unit (GU) of GC tube reaches ≥400 within the timed protocol and the GU of the drug tube is 100 or more (if 1% or more of the test population grows in the presence of the critical concentration of the drug).
- An isolate is defined as "**Susceptible**": when the growth unit (GU) of GC tube reaches ≥400 within the timed protocol and the GU of the drug tube is less than 100 (if there is no growth or less than 1% of the test population grows in the presence of the critical concentration of the drug).

10.2.3. GenoType® MTBDRplus assay and GenoType® MTBDRsl assay

Principle

The GenoType MTBDRplus test is based on the DNA STRIP technology and permits the molecular genetic identification of the *M. tuberculosis complex* and its resistance to rifampicin and/or isoniazid from cultivated samples or pulmonary smear-positive direct patient material. The whole procedure is divided into three steps: DNA extraction from cultured material (Lowenstein-

Jensen medium/liquid medium) or direct materials (pulmonary, smear–positive, and decontaminated), a multiplex amplification with biotinylated primers, and a reverse transcriptase. The result of the test can be easily read from the band pattern.

The GenoType MTBDRsl test is based on the DNASTRIP technology which is designed to improve the speed of diagnosis for drug resistant-TB, especially XDR-TB. Resistance to drugs develops through sequential accumulation of mutations in genes targeted by the respective drugs. Several genes were linked to resistance to TB drugs. GenoType MTBDRsl test uses the most known genes like rrs, gyrA, gyrB, and eis. Mutations in specific codons were identified and used for detecting resistance to specific drugs. Genotype MTBDRsl test consist of three steps: DNA extraction from decontaminated sputum specimens or cultured material (solid/liquid medium), a multiplex amplification with biotinylated primers, and a reverse hybridization.**DNA extraction**

Principle

DNA extraction is a procedure whereby DNA is fetched from bacterial cells or fragments of bacterial cells to be used for molecular biology analysis. With the GenoLyse chemical method test, the bacterial cells in the decontaminated patient sample or culture samples are chemically broken to expose the DNA by using a lyses buffer. The principle and procedure of DNA extraction for MTBDRplus assay and MTBDRsl assay is similar.

Procedure

1. Work on no more than 10 liquid culture isolates and 2 controls at a time.
2. Assemble isolates for the batch in the BSC.
3. Vortex thoroughly for 1 minute each of the liquid culture isolate and leave for 10-15 minute to settle the large clumps.
4. Using a sterile disposable Pasteur pipette, transfer 1 ml of each thoroughly mixed liquid culture isolate to labelled 1.5ml screw cap tube.
5. Centrifuge for 15 minutes at 10,000 RCF or 10263RPM using a micro-centrifuge with aerosol-tight rotor.
6. Unload tubes from the microcentrifuge and carefully carry the tubes to the BSC.
7. Discard supernatant from each tube by using 1000µl adjustable pipette.
8. Resuspend each pellet in 100µl Lysis Buffer (A-LYS).

9. Mix the contents of each tube using a sterile tip followed by thorough vortexing for at least 15 to 20 seconds.
10. Incubate the tubes for 5 minutes at 95 °C in a thermo-block.
11. Add 100 µl Neutralization Buffer (A-NB) and vortex the sample for 5 seconds.
12. Load the tubes into a microcentrifuge and spin for 5 minutes at 13,000 x g.
13. Carefully carry the tubes to the BSC. Uncap tubes one at a time, and transfer 100µl of DNA-containing supernatant to a sterile 1.5ml screw cap tube.
14. Store DNA at 2-8 °C for not more than 7 days. For longer storage, keep at -20 °C.

Amplification

All reagents needed for amplification such as polymerase and primers are included in the Amplification Mixes A and B (AM-A and AM-B) and are optimized for the tests.

1. After thawing, spin down AM-A and AM-B briefly and mix carefully by pipetting up and down.
2. Determine the number of samples (number of samples to be analyzed plus control samples) plus one contingency.
3. Prepare the number of sterile screw cap tube of 1.5ml in according to the number of samples and controls.
4. Label PCR tubes accordingly.
5. Prepare a master mix containing AM-A and AM-B and mix carefully but thoroughly (do not vortex) only in a room free from contaminating DNA.
6. Aliquot of 45 µl amplification mix into each labelled PCR tubes and add 5 µl molecular biology grade water to the negative control aliquot.
7. Ensure all PCR tubes are tightly closed.
8. Add 5 µl DNA to each aliquot except the negative control. The DNA solution should be added in a separate working area.

Hybridization

Pre warm shaking TwinCubator to 45°C; the maximum tolerated deviation from the target temperature is +/- 1°C. Dilute the conjugate concentration (CON-C, orange) and the substrate concentrate (SUB-C, yellow) with the corresponding buffer (i.e. CON-C with CON-D and SUB-C with CON-D) using a suitable tube in a dilution of 1:100 in the required amount needed. Mix

well and bring to room temperature for each stripe, add 10 µl concentrate to 1ml of the respective buffer. Dilute CON-C before each use. Diluted CON-C is stable for four weeks if stored at room temperature and protected from light.

Procedure

1. Pre-warm the Hybridization buffer (HYB, green) and Stringent buffer (STR, red) to 37-45°C in water bath (a total of 15 min). The reagent must be free from precipitate.
2. Pre-warm the Rinse solution (RIN) and sterile distilled water to room temperature.
3. Remove the DNA strips from the tube (shake strips down to end of tube, then carefully hold the end of the strips with forceps to remove them from the tube) and mark them with the pen provided.
4. Dispense 20 µl of the Denaturing solution (DEN, blue) to one corner of each of the wells of tray to be used.
5. Add 20 µl of the amplified DNA sample into the tray containing the DEN solution, thoroughly mix by pipetting upwards and downwards and incubate at room temperature for 5 minutes.
6. Carefully add 1 ml of pre-warmed HYB (green) to each well in the opposite end of DEN/DNA mixture using a single tip for each.
7. Gently tilt and shake the tray to homogenize the colour of the solution and do not splash the mixture or contaminate the neighbouring well.
8. Place the labelled strip facing upwards in each well and make sure the strips are completely covered with the solution in the well and re-position the strips if they turn over.
9. Place tray on TwinCubator and incubate for 30 minutes at 45°C by pressing the “START” of the TwinCubator. From this point, press the right arrow of Twincubator for procceding to the next steps in protocol.
10. Completely aspirate HYB solution (green) by pipetting with individual sterile pipette tip or sterile disposable Pasteur pipette into a small plastic discard container containing undiluted bleach solution. Change the pipette tips or Pasteur pipettes between each wells. Wipe off the condensation from the Twincubator lid before every incubation step.
11. Add 1ml of pre-warmed STR solution (red) to each well and incubator for 15 minutes at 45°C onTwinCubator by pressing the right arrow.
12. Completely aspirate the STR solution (red) as previously described for HYB removal. From this step forward work at room temperature form.

13. Add 1 ml of RIN solution to each well and wash for 1 minute by incubating at room temperature on TwinCubator.
14. Pour out the RIN solution completely from the into a small plastic discard container containing undiluted bleach solution.
15. Add 1 ml of diluted conjugate solution (CON) to each well and incubate for 30 minutes at 37°C using the shaking platform of TwinCubator.
16. Completely aspirate the CON solution using Pasteur pipette.
17. Add 1 ml of RIN solution to each well; wash for 1 minute by incubating at room temperature on TwinCubator.
18. Completely aspirate the RIN solution using Pasteur pipette.
19. Reapeat steps 17 and 18 respectively.
20. Add 1 ml of distilled water to each well; wash for 1 minute at room temperature while shaking TwinCubator and then compeletly remove the distilled water.
21. Add 1 ml of diluted substrate to each well and incubate for maximum of 10 minutes on Twincubator without shaking (cover the Twincubator with aluminum foil to protect the light). If colour reaction is too weak after completion of 4 to 5 minutes, replace the foil and re-incubate for several more minutes, up to a maximum of 10 minutes.
22. Stop reaction by briefly rinsing twice for 1 minute with distilled water and remove the distilled water after each wash.
23. Remove the strips from the tray using forceps and transfer to absorbent paper and air dry them.
24. Attach the strips to the results sheet using clear adhesive tape and interperate the results following instruction of the manufacturer.

Note that: the amplification and hybridization procedure of MTBDRplus assay and MTBDRsl assay are the same except using separate reagents which are provided with each kit.

10.1.4. Spoligotyping

Spoligotyping is a PCR-based method that simultaneously detect and type *Mycobacterium tuberculosis* complex bacteria.

It also contains the method for stripping the sample from the membrane and makes it ready for reuse, and the method for spoligotyping of one membrane (i.e. 38 diagnostic samples + 5

controls) and it is also possible to hybridise two membranes simultaneously. The method includes DNA isolation, amplification of DNA, hybridization with PCR product, detection and stripping of the membrane.

DNA Isolation

Principle

The DNA extraction is a procedure whereby DNA is fetched from bacterial cells or fragments of bacterial cells to be used for molecular typing of the bacteria. With the heat treatment, the bacterial cells in the decontaminated patient sample or culture samples are chemically broken to expose the DNA by using a heat treatment.

Procedure

1. Work on no more than 10 liquid culture isolates and 2 controls at a time.
2. Assemble isolates for the batch in the BSC.
3. Vortex thoroughly for 1 minute each of the liquid culture isolate and leave for 10-15 minute to settle the large clumps.
4. Using a sterile disposable Pasteur pipette, transfer 1 ml of each thoroughly mixed liquid culture isolate to labelled 1.5ml screw cap tube.
5. Heat the 1 ml isolate suspension from liquid culture at 80^{o}C using heat block or water bath for 45 minutes.
6. Sonicate the suspension for 15 minutes using sonicator.
7. Centrifuge the suspension at 13,000 rpm for 10 minutes at +4^{o}C.
8. Collect the supernatant into a new tube and store at -20^{o}C for molecular characterization.

In vitro amplification of spacer DNA by PCR

Principle

Amplification of the spacers is accomplished by using the primers DRa and DRb, which enable to amplify the whole DR region. Only a very small amount of template DNA is required. Typically the PCR is performed on 10 ng purified chromosomal Mycobacterial DNA but, with minor

adaptations, DNA extracts from clinical samples or lysed bacteria (from freezer or Lowenstein) can also serve as template. The PCR products are labeled with biotin, because one of the primers is biotinylated. The primers for the PCR are based on the DR sequence:

DRa: 5'-GGT TTT GGG TCT GAC GAC-3', biotinylated at 5' end.

DRb: 5'-CCG AGA GGG GAC GGA AAC-3'

Procedure

1) Always include chromosomal DNA of M. tuberculosis strain H37Rv and M. bovis BCG P3 as positive controls. Use water as a negative control.
2) Prepare the reaction mixture:
   2 µl template DNA
   3 µl primer DRa (0.2 µmol/µl)
   3 µl primer DRb (0.2 µmol/µl)
   20 µl 2×TaqPCR Master-Mix
   12 µl MQ water (to a final volume of 40 µl)
3) Place the tubes in a PCR device for amplification, and run the PCR using the following program:
   3 min 96°C 1 Cycle
   1 min 96°C 20 cylces
   1 min 55°C
   30 sec 72°C
   5 min 72°C 1 Cycle
   ∞ 4°C

Preparation of reagents for hybridization

Primary buffer (2XSSPE/ 0.1SDS): used to dilute PCR product and pre-warm the membrane.

250 ml/round=25 ml 20XSSPE + 222.5 ml distill water + 2.5 ml 10%SDS

Split the primary buffer with two bottles; 10 ml for PCR product dilution (store at room tempertature) and 240 ml for pre-warm the membrane (equilibrate at 60°C).

Secondary buffer (2XSSPE/ 0.5SDS): used to remove unbound PCR product and residual POD

1000 ml/round=100 ml 20XSSPE + 850 ml distill water + 50 ml 10%SDS

Split the secondary buffer into two bottles of each 500 ml and equilibrate the first bottle at 60°C and the second bottle at 42°C.

2XSSPE: used to remove excess SDS and store at room temperature

500 ml/round=50 ml 20XSSPE + 450 ml distill water

Preparation of reagent for membrane washing and storage

1%SDS: used to remove the PCR product and equilibrate at 60°C

500 ml/round=50 ml 10%SDS + 450 ml distill water

20mM EDTA: used to wash and store the membrane and store at room temperature

500 ml/round=20 ml of 0.5EDTA + 480 ml of distill water

Hybridization with PCR product and detection

Purpose

Hybridization of the biotin-labeled PCR products to the immobilized spacer-oligos that represent spacers of known sequence. The presence of spacers is visualized on film as black squares after incubation with streptavidin-peroxidase and ECL-detection.

Note

All incubations should take place in a plastic container under gentle shaking, unless otherwise stated.

Thoroughly clean the miniblotter with soap and a dedicated brush before use.

Never touch the membrane with gloves, the powder caused background. Use forceps.

The quality of the SDS is of critical importance. It should be fresh, do not store it for longer than one week.

Procedure

1. All buffers should be pre-warmed before use. Prepare the following buffers from concentrated stocks, using demineralized water for dilution (quantities for one membrane):

   250 ml 2xSSPE/0.1% SDS at 60°C,

   500 ml 2xSSPE/0.5% SDS at 60°C,

500 ml 2xSSPE/0.5% SDS at 42°C,

500 ml 2xSSPE at room temperature.

2. Add 20 µl of the PCR products to 150 µl 2xSSPE/0.1% SDS.
3. Heat-denature the diluted PCR product for 10 min at 99°C and cool on ice immediately.
4. Wash the membrane for 5 min at 42°C in 250 ml 2xSSPE/0.1% SDS.
5. Place the membrane and a support cushion into the miniblotter, in such a way that the slots are perpendicular to the line pattern of the applied oligonucleotides.
6. Remove residual fluid from the slots of the miniblotter by aspiration.
7. Fill the slots with the diluted PCR product (avoid air bubbles) and hybridize for 60 min at 60°C on a horizontal surface (no shaking!). Avoid contamination of neighbouring slots.
8. Aspirate and remove the samples from miniblotter and take the membrane from the miniblotter using forceps.
9. Wash the membrane twice, for 5 min each in 250 ml 2xSSPE/0.5% SDS at 60°C.
10. Place the membrane in a rolling bottle and allow it to cool down to prevent inactivation of the peroxidase in the next step.
11. Add 7 µl streptavidin-peroxidase conjugate (500 U/ml) to 14 ml of 2xSSPE/0.5% SDS, and incubate the membrane in this solution for 45 to 60 min at 42°C in the rolling bottle.
12. Wash the membrane twice, for 10 min each in 250 ml of 2xSSPE/0.5% SDS at 42°C.
13. Rinse the membrane twice, for 5 min each in 250 ml of 2xSSPE at room temperature.

Detection procedure (development of the film)

1. For chemiluminiscent detection of hybridizing DNA, prepare ECL by adding 10 ml of solution 1 and 10 ml of solution 2 and then immerse the membrane for 1 to 2 min in the 20 ml ECL detection liquid (ensure the membrane is completely covered in the ECL solution).
2. Cover the membrane with a transparent plastic sheet or Saran-wrap and expose a light sensitive film to the membrane using Autorad cassette and place a new ECL film on top.
3. Close the cassette, wrap in a black sack and leave in a drawer to expose for 10 to 20 minutes (depending on the intensity of the black square).
4. Ensure that the film developer and fixer is stored at the temperature of 22°C . Pour of the film developer (100 ml), water (100 ml) and fixer (100 ml) into three separate trays (20x25cm); the film developer on the left, water on the middle and fixer on the right sequentially.

5. Turn on the infrared safety light (red light), remove the autorad from the cassette and place the autorad in the film developer ensuring that the Autorad is entirely submerged.
6. Tip gently until an image of black square is visible- approximately 2 minutes.
7. Rinse the Autorad in distilled water for 5 to 10 sec.
8. Transfer the Autorad to the tray containing the fixer, ensure the Autorad is entirely immersed by agitating for 1 to 2 min.
9. Leave for 1 minute, rinse in distilled water for 5-10 sec.
10. Discard the developer but transfer fixer into a labeled dark bottle. The fixer can be used for maximum 5 times (or up to one month).

Note

If the signal is too weak or too strong the membrane can be used again directly to expose another film for a shorter or longer period.

Stripping or regeneration of the membrane

Purpose

The hybridized PCR product is dissociated from the membrane to regenerate the membrane for the next hybridization. A membrane can be regenerated for at least 10 times.

Procedure

1. Unwrap the membrane and place in a plastic container
2. Wash the membrane twice with 250 ml 1% SDS by incubation at 80°C for 30 min while shaking.
3. After the second wash, leave at room temperature for 5 minutes to cool before discarding the liquid, and then wash the membrane in 20 mM EDTA pH 8, for 5 minutes at room temperature.
4. Discard the liquid and refresh with another 250 ml 20 mM EDTA and store the membrane at 4°C until use (sealed in plastic or wrapped in Saran-wrap, to avoid dehydration of the membrane).

<u>Annex 10.2:</u> Data collection form for Molecular epidemiology and drug resistance pattern of *Mycobacterium tuberculosis* isolated from Amhara, Gambella and Benishangul-Gumuz regions of Ethiopia

Individual ID Number: _____			
Demographic and Medical data			
	Region	_____	
	Age	_____ (in Years)	
	Sex	☐ Male ☐ Female	
	Signs and symptoms of TB?	Fever?	☐ Yes ☐ No
		Chronic cough more than 2 weeks?	☐ Yes ☐ No
		Night sweating	☐ Yes ☐ No
		Weight loss	☐ Yes ☐ No
		Indicative chest X-ray finding	☐ Yes ☐ No
		Others, specify?	_____
	Was patient given diagnosis of pulmonary TB?	☐ Yes ☐ No	
	Contact History with known **<u>TB</u>** patient?	☐ Yes ☐ No	
	Contact History with known **<u>MDR-TB</u>** patient?	☐ Yes ☐ No	
	Was there a previous history of TB?	☐ Yes ☐ No	
	Previous exposure of first-line anti-TB drugs (>1 month)?	☐ Yes ☐ No	
	If YES to the above question	☐ Relapse ☐Treatment after failure	

		☐Treatment after loss to follow-up ☐Other previously treated	
	Was patient an MDR-TB and now on MDR treatment?	☐ Yes ☐ No	
	Is the patient health care worker?	☐ Yes ☐ No	
	Co-infection?	☐ Yes ☐ No	☐ HIV ☐ Diabetes Mellitus (DM) ☐ Others, specify _____ _____
	The patient is classified as	☐Presumptive TB patient ☐Presumptive MDR-TB patient ☐MDR-TB follow-up patient at month _____	
Laboratory result from isolate			
	Name of the TB reference laboratory: _____		
	Date sputum samples received by TB reference laboratory:____/____/_____ DD MM YYYY		
	LJ Culture		
	LJ result (check boxes)	☐ MTBC positive ☐ NTM Date detected: Date detected: ____/____/_____ ____/____/_____ DD MM YYYY DD MM YYYY ☐ MTBC negative ☐ Contaminated ☐ MTBC positive with contaminants	

	If MTBC positive, grading	☐Actual count (1-9 colonies) ☐1+ (10 - 100 colonies) ☐2+ (>100 - 200 colonies) ☐3+ (> 200 colonies)	
	BACTEC MGIT culture		
	BACTEC MGIT culture results (check boxes)	☐ MTBC positive ☐ NTM Date detected: Date detected: ____/____/_____ ____/____/_____ DD MM YYYY DD MM YYYY ☐ MTBC negative ☐ Contaminated ☐ MTBC positive with contaminants	
	Sub-culture with MGIT		
		☐ MTBC positive ☐ MTBC positive with contaminants ✓ Re-decontamination ☐ performed ☐ Not performed ☐ MTBC negative ☐ Contaminated	
	HAIN molecular assay		
	Date of testing	____/____/_____ DD MM YYYY	
	MTBDRplus	**Wild type missing** ☐ rpoBWT1 ☐ katG WT ☐ rpoBWT2 ☐ inhA WT1 ☐ rpoBWT3 ☐ inhA WT2 ☐ rpoBWT4 ☐ rpoBWT5 ☐ rpoBWT6	☐MTB RIF ☐ Sen ☐ Res INH ☐ Sen ☐ Res ☐ Non MTB detected ☐ MTB not detected ☐ Invalid

		□ rpoBWT7 □ rpoBWT8 **Mutation** □ rpoBMUT1 □ inhAMUT1 □ rpoBMUT2A □ inhAMUT2 □ rpoBMUT2B □ inhAMUT3A □ rpoBMUT3 □ inhAMUT3B □ katGMUT1 □ katGMUT2	
	MTBDRsl	**Wild-type missing** □ gyrAWT1 □ eisWT1 □ gyrAWT2 □ eisWT2 □ gyrAWT3 □ eisWT3 □ gyrBWT □ rrsWT1 □ rrsWT2 **Mutation** □ gyrAMUT1 □ gyrBMUT1 □ gyrAMUT2 □ gyrBMUT2 □ gyrAMUT3A □ rrsMUT1 □ gyrAMUT3B □ rrsMUT2 □ gyrAMUT3C □ eisMUT1 □ gyrAMUT3D	□ MTB AM □ Sen □ Res CM □ Sen □ Res KM □ Sen □ Res OFx □ Sen □ Res □ Non MTB detected □ MTB not detected □ Invalid
	Phenotypic drug sensitivity test		
	First line drugs	STR □ Sen □ Res INH □ Sen □ Res RIF □ Sen □ Res EMB □ Sen □ Res PZA □ Sen □ Res	

	Second line drugs	AM □ Sen □ Res CM □ Sen □ Res Cfx □ Sen □ Res Eto □ Sen □ Res KM □ Sen □ Res Lvx □ Sen □ Res Lz □ Sen □ Res MFx □ Sen □ Res OFx □ Sen □ Res
	Spoligotyping	SIT no: _____ Family: _____ Lineage: _____ □ Unkown Lineage: _____

Data Collection Form Reviewed by: _____

(for study staff only) **Printed name**

_____

Signature

_____

Date

Annex 10.3: Assurance of Principal Investigator

I the undersigned Bazezew Yenew, hereby confirm that this thesis is my original work and has not previously submitted and presented at any university for the fulfilment of a degree.

Signature: _____

Date: _____

The thesis has been submitted for examination with my approval as university advisor.

Name: Woldearegay Erku Abegaz, Ph.D., Associate Professor

Signature: _____

Date: _____

The thesis has been submitted for examination with my approval as external advisor.

Name: Abebaw Kebede, PhD candidate

Signature: _____

Date: _____