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Full Length Research Paper

Drug susceptibility test on *Mycobacterium tuberculosis* isolated from pulmonary tuberculosis patients in three sites of Ethiopia

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The spread of multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis* has become a challenge to the global tuberculosis control and prevention program. Nevertheless, no adequate information has been made available on the drug susceptibility status of *M. tuberculosis* in Ethiopia. Hence, this study aimed at evaluating the drug resistance patterns of *M. tuberculosis* in the three sites of Ethiopia and its association with socio-demographic factors. A total of 102 *M. tuberculosis* isolates obtained from tuberculosis (TB) patients from three different sites were tested for drug susceptibility against the first and second-line anti-TB drugs using the proportion method on enriched Middle Brook 7H10 agar. The result showed that 36.3% of the isolates were resistant to at least one of the first line anti-TB drugs used in this study. The proportions of mono-resistance were 29.4, 13.7, 13.7 and 23.5% to isoniazid, rifampicin, streptomycin and ethambutol, respectively. The proportion of MDR *M. tuberculosis* was 11.8%. Only 55% of the isolates were susceptible to all the second line anti-TB drugs used in this study. One isolate (1%) was extensively drug-resistant (XDR). The occurrence of MDR *M. tuberculosis* isolates was not associated with sex, age, anti-TB treatment history, HIV status and location. The findings of this study showed moderate number of MDR *M. tuberculosis* isolates in the study area, warranting the necessity of the surveillance and monitoring.

Key words: *Mycobacterium tuberculosis*, drug resistance, Ethiopia.

INTRODUCTION

Tuberculosis (TB) is a major public health problem world-wide claiming the lives of 20 individuals per 100, 000 population with 164 cases per 100,000 every year (Pedro et al., 2007; WHO, 2009b). In Ethiopia, TB is the leading cause of morbidity, third cause of hospital admission and second cause of death next to malaria (FMOH, 2008). Ethiopia ranks seventh among the world's 22 high-burden TB countries suffering 80% of the global case burden.

According to the World Health Organization's (WHO's) Global TB Report 2009, the country had an estimated 314,267 TB cases in 2007, with an estimated incidence rate of 378 cases per 100,000 population.

Drug treatment is fundamental for controlling TB, promoting the cure of the disease and breaking the chain of transmission when the anti-TB drug regimen is completely and correctly followed. The two aims of TB treatment are to interrupt the transmission by rendering patients noninfectious and to prevent morbidity and death by curing patients with TB (Pedro et al., 2007). However, the spread of MDR strains of *Mycobacterium tuberculosis*

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has become a major public health concern challenging the global TB control and prevention program since these bacteria often cause incurable disease. The MDR *M. tuberculosis* is isolated not only from previously treated TB patients but also from new cases due to transmission in the community (Kristian et al., 2002; Kim, 2005). WHO Global Project on TB drug resistance surveillance showed a median prevalence of 1.8% of MDR *M. tuberculosis* strains globally (WHO, 2000).

Thus, drug resistant *M. tuberculosis* strains threaten the national TB control program and the major problem is MDR *M. tuberculosis* strains (WHO, 2009b). Effectiveness of a standard anti-TB treatment regimen correlates with the *in vitro* drug susceptibility pattern of the infecting tubercle bacilli. As a result, the drug susceptibility test including novel strategies has become more important than ever; and yet, there is a shortage of information in Ethiopia regarding the susceptibility status of *M. tuberculosis* to first line and second line anti TB drugs which determines the treatment and control of TB. The aim of the present study was to evaluate the drug susceptibility pattern of *M. tuberculosis* isolates.

MATERIALS AND METHODS

Setting and study isolates

A laboratory based study was conducted from February to July, 2011 on 102 *M. tuberculosis* isolates randomly selected from 300 sputum smear positive (AFB positive) pulmonary TB patients using computer generated random numbers of isolates. They were isolated from sputum smear positive (AFB positive) pulmonary TB patients that attended three health institutions of the three sites: Bahir Dar (Northwest Ethiopia), Ambo (Western Shoa Ethiopia) and Fitche (Central Ethiopia) during the study period. The sputum sample was cultured on Lowenstein Jensen (LJ) medium. Then the colonies were collected and Ziehl-Neelsen (AFB) staining was done for the presence of the Acid Fast Bacilli (AFB) in the colonies according to WHO guide line (WHO, 2004). DNA was extracted by heat killing of the colonies and Region of Deference 9 (RD9) deletion typing was performed by standard thermocycler (VWR Thermocycler, United Kingdom) for confirmation of the presence or absence of RD9 using primers; RD9Ff 5'-GTG TAG GTC AGC CCC ATC C-3', RD9Fr 5'-GCC CAA CAG CTC GAC ATC-3' and RD9Itn 5'-CTG GAC CTC GAT GAC CAC TC-3' as described previously (Huard et al., 2003). Interpretation of the result was made on the basis of detection of bands of different sizes, that is, when a band size 396 bp was observed, the isolate was considered as *M. tuberculosis* while when a band size of 575 bp was observed the isolate was considered as either *Mycobacterium bovis* or *Mycobacterium africanum* (Brosch et al., 2002; Smith et al., 2006). For quality control, *M. tuberculosis* H37Rv, *M. bovis* BCG and water were included.

Data collection

The demographic data of the patients including age, gender, previous anti-TB treatment history and the HIV status was determined by rapid HIV screening method at the Providing Initiative Testing and Counseling (PITC) clinic by nurses and the data was collected from the hospital record in the respective study sites.

Evaluation of the isolates for susceptibility to anti-TB drugs

First line anti-TB drugs (FLD) including isoniazid, streptomycin, rifampicin and ethambutol (Sigma Chemicals Co., Germany) were used to prepare the stock solutions required for preparing drug containing media. Stock solutions (1 mg/ml) of isoniazid, streptomycin and ethambutol were prepared in sterile distilled water, whereas rifampicin was prepared in di-methyl sulphoxide (DMSO), and the recommended critical concentrations (CC) were 2, 0.2, 1.0 and 5 µg/ml, respectively (Kent and Kubica, 1985). The SLD considered in this study were *P*-aminosalicylic acid (PAS) and capromycin (polypeptide), amikacin and kanamycin (aminoglycosides), ethionamide; ofloxacine and moxifloxacine (fluoroquinolones) (Sigma chemicals Co, Germany) with recommended CC of 2, 10, 1, 2, 5, 5, 2 and 1 µg/ml, respectively (WHO, 1997). All were prepared in concentration of 1 mg/ml in sterile distilled water except moxifloxacine which was prepared in the same concentration in NaOH for its stock solution preparation (Kent and Kubica, 1985). The stock solution was further diluted to prepare a CC for each drug in a limited volume of the 7H10 media in the 24-well plates to maintain the recommended CC of each drug.

The enriched middle brook 7H10 agar based medium was prepared according to Van Soolingen et al. (2007) guide line. For the preparation of bacterial suspension, a loop full of mycobacterial colony from the culture was collected and emulsified in a test tube containing about 2 ml of sterile distilled water; the solution was vortexed and allowed to stand for the coarse particles to settle down. The mycobacterial solution was carefully decanted to other clear, sterile test tubes, and the opacity/turbidity was matched with McFarland standard No. 1 (3×10^8 CFU/ml) by adding sterile distilled water drop by drop. This suspension was distributed in 10 µl volumes into 11 wells of the 24-well plate containing 7H10 medium with a precise concentration of the selected anti-TB drugs and into the 12th well containing drug free medium. The 13th well of each plate was inoculated with a mycobacterial solution containing 1% of the suspension (10 µl of the suspension that matches the McFarland standard No.1 was taken and diluted by 990 µl of sterile distilled water in a sterile test tube to obtain the 1:100 suspensions). This was followed by incubation at 35°C in the presence of sufficient humidity.

Reading and interpretation of sensitivity

After 2 to 3 days of incubation, the plates were read to see if there was any contamination and mixtures with other bacteria. Thereafter, they were incubated for 12, 19 and 28 days and checked for the growth of bacteria. Bacterial growth in the wells containing drugs was compared with the controls. The CC (break point) was used to determine whether a strain is sensitive or resistant. When there was growth above this breaking point, the strain was considered as resistant. The sensitivity result of the isolates were read by visual comparison of the drug containing media (1:1 bacterial suspension) with the drug free control on which 1:100 bacterial suspensions were inoculated.

The two concentrations of bacterial suspension (1:1 and 1:100) inoculated on the two well containing media had a factor of 100 between them. Equal growth on the two media, in principle, would mean 1% of the isolates are resistant to that particular drug. However, for reason of accuracy, the test was repeated where the growth became equal or nearly equal for both. MDR-TB was defined as isolates resistant to at least RIF and INH. An isolate can be labeled as XDR if resistant to one of these drugs and to one of the quinolones (e.g. ofloxacin or moxifloxacin) in addition to being MDR (Moore, 2007; Banjere et al., 2008). Internal and external quality controls were conducted through the whole study period. Drug susceptibility testing procedures (WHO, 2008a) were adhered to

Table 1. Drug resistance pattern of study isolates to first line anti TB drugs.

Region	Isolates	Susceptible to all drugs No. (%)	Resistant				
			At least one drug n (%)	INH No. (%)	RIF No. (%)	ETB No. (%)	SM No. (%)
Bahir Dar	35	28(80.0)	7(20.0)	6(20.0)	3(21.4)	6(25.0)	2(20.0)
Fitche	33	22(66.7)	11(33.3)	7(23.3)	5(35.7)	7(29.17)	1(6.7)
Ambo	34	15(44.1)	19(55.9)	17(56.7)	6(42.9)	11(45.8)	11(73.3)
Total	102	65(63.7)	37(36.3)	30(29.4)	14(13.7)	24(23.5)	14(13.7)

INH = Isoniazid, RIF = rifampicin, SM = streptomycin, ETB = ethambutol.

Table 2. Drug resistance pattern of study isolates to second line anti TB drugs.

Region	Isolates	Susceptible to all drugs no. (%)	Resistant							
			At least one drug	CAP No. (%)	AMK No. (%)	KAN No. (%)	OFX No. (%)	MOX No. (%)	PAS No. (%)	ETH No. (%)
Bahir Dar	35	25(71.4)	10(28.6)	1(2.9)	1(2.9)	1(2.9)	3(8.6)	3(8.6)	3(8.6)	5(14.3)
Fitche	33	16(48.5)	17(51.5)	2(6.1)	1(3.0)	2(6.1)	1(3.0)	1(3.0)	5(15.2)	10(3.0)
Ambo	34	15(44.1)	19(55.9)	9(26.5)	6(17.6)	7(20.6)	2(5.9)	1(2.9)	9(26.5)	12(35.3)
Total	102	56(54.9)	46(45.1)	12(11.8)	8(7.8)	10(9.8)	6(5.9)	5(4.9)	17(16.7)	27(26.5)

AMK = Amikacin, CAP = capromycin, KAN = kanamycin, OFX = ofloxacin, MOX = moxifloxacin, PAS = P-aminosalicylic acid, ETH = ethionamide.

while conducting the test. Standard H37Rv laboratory strain was included in each batch of culture.

Data analysis

Data were entered into Excel Microsoft 2007 and the proportion of mono- and multi-drug resistance among new and previously treated TB cases was calculated. Multivariate logistic regression analysis was performed using the STATA statistical software (version 8.0) to identify the associated factors for MDR-TB. Statistical significance was assessed at a two tailed P value of 0.05.

Ethical clearance

This study was approved by Institutional Review Board of Aklilu Lemma Institute of Pathobiology, Addis Ababa University, Addis Ababa, Ethiopia.

RESULTS

All 102 isolates found to be *M. tuberculosis* were recovered from 52 (51%) male and 50 (49%) female patients with the majority of them from 15 to 45 years age category. Ninety three (91.2%) were previously untreated for TB, while 9 (8.8%) were treated cases and 8 (7.8%) were HIV positive.

The result showed that 36.3% isolates were resistant to at least one of the first line anti-TB drugs. The proportions of mono-resistance were 29.4, 13.7, 13.7 and 23.5% for isoniazid, rifampicin, streptomycin and ethambutol, respectively (Table 1).

The susceptibility to the three injectable second line drugs (amikacin, capromycin and kanamycin) is important in defining the degree of resistance of an isolate. The observed mono-drug resistance to the three injectable second line drugs was 11.8% for capromycin, 7.8% for amikacin and 9.8% for kanamycin. Floroquinolones showed the least proportion of mono-resistance: 5.9% for ofloxacin and 4.9% for moxifloxacin (Table 2).

The pattern of MDR *M. tuberculosis* isolates is presented in Table 3. The overall proportion of MDR isolates was 11.8% and highest (17.6%) at Ambo (Western Shoa, Ethiopia) while one (8.3%) isolate, out of the 11.8% MDR *M. tuberculosis,* was XDR and was found in Bahir Dar (North-West Ethiopia).

The occurrence of MDR *M. tuberculosis* strains was not associated with age, sex, study town, previous anti-TB treatment and history of HIV sero-status of the patient used as source of the strains (Table 4). Nonetheless, the absence of such association could be attributed to the low number of study isolates that were used for this study.

DISCUSSION

The present study showed that resistance to the first and second line drugs were 36.3 and 45.1%, respectively, on the average. Previous studies done in the country at different places reported different types of resistance to the first line drugs. For example, 15.1% resistance to the first line drug was reported in Bahir Dar (Mekonnen et al., 2010) while 73.9% resistance to the second line drug was

Table 3. Proportion of MDR *M. tuberculosis* isolates in the study regions.

Region	Isolates	Drug resistance			
		HR	HRE	HRS	HRSE
		Number (%)	Number (%)	Number (%)	Number (%)
Bahir Dar	35	3 (8.6)	3(8.6)	2(5.7)	2(5.7)
Fitche	33	3(9.1)	3(9.1)	1(3.03)	1(3.03)
Ambo	34	6(17.6)	5(14.7)	5(14.7)	5(14.7)
Total	102	12(11.76)	11(10.78)	8(7.84)	8(7.84)

HR = Isoniazid, rifampicin; HRE = isoniazid, rifampicin, ethambutol; HRS = isoniazid, rifampicin, streptomycin; HRSE = isoniazid, rifampicin, streptomycin, ethambutol.

Table 4. Effects of different patients related factors on the occurrence of MDR *M. tuberculosis* isolates as analyzed using logistic regression.

Factor	Isolate	Number of MDR (%)	Crude odds ratio (95% CI)	P -value	Adjusted odds ratio (95% CI)	P- value
Sex						
Male	52	5(9.6)	1		1	
Female	50	7(14)	1.53(0.45-5.18)	0.494	1.67(0.46-6.02)	0.429
Age (year)						
<15	3	1(33.3)	1		1	
15-45	83	9(10.8)	0.24(0.02-2.95)	0.267	0.26(0.02-3.75)	0.326
>45	16	2(12.5)	0.28(0.02-4.79)	0.384	0.33(0.02-6.20)	0.456
Anti-TB treatment history						
Untreated	93	11(11.7)	1		1	
Treated	9	1(11.11)	0.93(0.10-8.17)	0.949	1.28(0.13-12.22)	0.825
HIV-sero status						
Non reactive	94	11(55.6)	1		1	
Reactive	8	1 (12.5)	1.07(0.12-9.60)	0.946	0.95(0.09-9.40)	0.967
Location						
Bahir Dar	35	3(8.57)	1		1	
Fitche	33	3(9.09)	1.06(0.19-5.70)	0.940	1.30(0.23-7.37)	0.760
Ambo	34	6(17.65)	2.28(0.52-9.99)	0.272	2.38(0.51-11.09)	0.268

MDR = Multidrug resistant, CI = confidence interval.

first line drug was reported in Bahir Dar (Mekonnen et al., 2010) while 73.9% resistance to the second line drug was reported in Addis Ababa (Agonafir et al., 2010). first line drug was reported in Bahir Dar (Mekonnen et al., 2010) while 73.9% resistance to the second line drug was reported in Addis Ababa (Agonafir et al., 2010). According to most recent global finding, resistance was found to be 0 (Europe) to 56.3% (Azerbajan). In the present study, the numbers of MDR TB were 11.7% in untreated cases and 11% in treated cases. This proportion is different from that of earlier reports in Addis Ababa, which recorded 2.3% in untreated cases and 71% in treated cases (Abate et al., 1998; Agonafir et al., 2010). This difference could be attributed to the significant number of cases difference between untreated and treated cases. However, the overall proportion of MDR recorded by the present study is similar to that reported in Addis Ababa earlier (Abate et al., 1998; Agonafir et al., 2010). Globally, the proportion of MDR-TB ranged from 0% in eight countries to 19.4% in the Republic of Moldova and 22.3% in Baku City, Azerbaijan, and 2.9% worldwide (WHO, 2008b).

On the other hand, the WHO 2007 estimate of MDR for Ethiopia is 1.6% in new cases and 12% in treated cases

(WHO, 2009a). Although, the result of the present study is closer to this value, the size of the samples per study site was inadequate to comfortably state the prevalence of MDR in the respective sites but the results of this study may reflect the underlying danger of public threat related to TB management and control. The study also warrants the necessity of conducting similar studies on a large scale to fully capture the picture of the problem for information based interventions.

Although, it is recommended to test the isolates in second line drugs after they are confirmed to be MDR for the first line drugs (APHL, 2007), it was not possible to do so in the present study because of the short time given to complete this study. XDR-TB is a form of TB that are resistant to the most effective anti-TB drugs and some consider that XDR-TB strains have emerged from the mismanagement of MDR-TB and once created can spread from one person to another (Pillay and Sturm, 2007). Out of the total 12 MDR-TB isolates, one isolate was thought to be XDR-TB which is lower as compared to the earlier reports that showed 4.4% (Agonafir et al., 2010) and 12% (Mohamad et al., 2006). Therefore, this indicated that, the TB control program should launch monitoring system for limiting the spread of XDR TB in the country.

Males are more commonly affected by TB than females in most countries and male or female TB patients could have different levels of risks for drug resistance due to differences in access to health-care services or exposure to other risk factors (Shao et al., 2011). Nonetheless, similar to a study by Bruchfeld et al. (2002), no association was observed in this study between the occurrence of MDR-TB and either the sex or the age of patients from which the isolates were recovered. The association between age and the risk of MDR-TB is not established in the literatures as different studies use different cut-off points for age groups although MDR-TB patients were more likely to be younger than 65 years (Shao et al., 2011).

There are controversial reports on the effect of infection with HIV on the occurrence of MDR TB, as some studies found increased risks of MDR TB among patients co-infected with TB and HIV (Ramaswamy and Musser, 1998) while others reported no association between the two (WHO, 2000; Bruchfeld et al., 2002). It was difficult to find association between co-infections and risk of acquiring MDR in the present study because of the very low number of known HIV positive cases met during the data collection.

As seen in many other studies, the history of anti-TB treatment has been consistently associated with the risk of MDR-TB and a systematic review on 29 studies in Europe reported that the pooled risk of MDR was up to 10 times higher in previously treated cases than in never treated ones (Shao et al., 2011). However, some studies showed no association between history of anti-TB treatment and risk of MDR-TB (Bruchfeld, et al., 2002). In this study, it was difficult to compare the risk of developing drug resistance between untreated cases and previously treated cases because of the relatively low number of previously treated cases.

The findings of this study showed moderate proportion of MDR *M. tuberculosis* isolates and the occurrence of XDR *M. tuberculosis* isolates in the study area, thereby warranting the surveillance and monitoring of such strains so that their transmission is curtailed.

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