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Full Length Research Paper

Primary resistance rates of *Mycobacterium tuberculosis* complex strains isolated from new tuberculosis cases: A 6-year observation

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The aim of the study was to evaluate the automated mycobacteria growth ındicator tube (MGIT) for drug susceptibility testing of *Mycobacterium tuberculosis* and to determine resistance patterns. We used BACTEC MGIT 960 System to determine the susceptibility of *M. tuberculosis* complex isolates to major anti tuberculous agents. Patients with single and first positive isolates were enrolled in the study. We have performed our drug susceptibility study between January 2005 and December 2010 for monitoring of drug resistance patterns in six years. A total of 1240 (77.16%) of the 1607 isolates were susceptible to all four of the antimycobacterial agents while 369 (22.96%) were found to be resistant to one or more of the drugs. The rate of isolates resistant to streptomycin (SM) was 6.84%, the other rates were 17.17% to isoniazid (INH), 5.28% to rifampicin (RIF) and 4.10% to ethambutol (ETM). Single drug resistance rates were found to be 12.13% for INH, 0.99% for RIF, 5.6% for SM, and 1.74% for ETM. The ratio of resistant isolates to all four drugs was 0.74% (n=12) and the prevalence of multidrug resistant isolates was 3.92% (n=63). It was concluded that resistance to INH and RIF continues almost in a straight line in the present study. Monitoring of drug resistance patterns is essential for accurate drug regimen in management of tuberculosis.

Key words: *Mycobacterium tuberculosis* complex, primary resistance, ısoniazid, rifampicin.

INTRODUCTION

The World Health Organisation estimated the global burden of tuberculosis disease in 2009 as 9.4 million incident patients, 14 million prevalent cases and 2.38 million deaths in the 2010 TB report (WHO/HTM/TB/2010-3; WHO/HTM/STB/2010-2). An estimated 11–13% of incident cases were HIV-positive. Among TB patients notified in 2009, an estimated 250, 000 had multidrugresistant TB (MDR-TB). There were an estimated 440,000 cases of MDR-TB in 2008. The four countries that had the largest number of estimated cases of MDR-TB in absolute terms in 2008 were China, India, the Russian Federation and South Africa. Primary resistance is the resistance pattern seen in new patients who have not previously been exposed to anti-TB drugs. Secondary resistance is the resistance pattern in patients with a previous history of anti-TB treatment and is due to ineffective chemotherapy. Fifty eight countries and territories have reported at least one case of extensively drug-resistant TB (XDR-TB). XDR-TB is defined as resistance to isoniazid and rifampicin (that is, MDR-TB) plus resistance to a fluoroquinolone and, at least, one second-line injectable agent (amikacin, kanamycin and/or capreomycin) (Bozkurt et al., 2010; EuroTB, 2007). Surveillance of primary and secondary resistance patterns is important in assessing the quality of chemo-therapy programs over several years and detecting errors in past treatments respectively. The resistant strains are characterised by a unique, lipid- containing and rigid core of the cell wall. The mycobacterial cell wall is less permeable to hydrophilic molecules than other

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Abbrevations: MGIT, Mycobacterium growth indicator tube; **MTC,** *Mycobacterium tuberculosis* complex; **TB,** tuberculosis; **INH,** ısoniazid; **RIF,** rifampicin; **ETM,** ethambutol; **SM,** streptomycin; **MDR-TB,** multi drug resistant tuberculosis; **XDR-TB,** extensively drug-resistant tuberculosis.

Table 1. Concentrations of drugs used in this study.

Drug	Concentration of drug after reconstitution (µg/mL)	Volume added to MGIT Tubes for test (µL)	Final concentration in MGIT tubes (µg/mL)
MGIT Streptomycin	83	100	1.0
MGIT Isoniazid	8.3	100	0.1
MGIT Rifampicin	83	100	1.0
MGIT Ethambutol	415	100	5.0

bacteria. That is why mycobacteria are resistant against the majority of drugs commonly used against bacteria (De Rossi et al., 2006). Anti tuberculous agents interfere with enzymes involved in cell wall biosynthesis (isoniazid, ethambutol, ethionamide), protein synthesis (streptomycin, other aminoglycosides, macrolides), transcription (rifampin), or DNA replication (quinolones). In *M. tuberculosis* spontaneous mutations occur at a frequency of approximately 10^{-5} to 10^{-8} (Inderlied and Nash, 1996). Since resistances to various drugs arise independently, the likelihood of spontaneous mutation to isoniazid and rifampin, for instance, is one in 10^{-14}. The threat of dual mutations is one of the rationales for why an anti-tuberculosis therapy should consist of a combination of drugs (Brennan and Draper, 1994). To be successful in the management of tuberculosis, rapidly detecting the susceptibility of MTC strains to first line drugs is very important (Aydın et al., 2011).

Many studies performed in Turkey confirmed that the resistance rates and patterns of MTC may show different characters in different regions (Talay et al., 2003; Taşova et al., 1997). We aimed in this study to observe the regional and current rates of primary drug susceptibilities for MTC strains in active pulmonary tuberculosis by using clinical specimens (bronchial fluid and sputum).

MATERIALS AND METHODS

American Center for Disease Control (CDC) recommends performing susceptibility tests on specimens, within, 28 to 30 days after they are submitted to a laboratory, as we have done in this study (CDCP, 1993).

Strains

1607 MTC strains isolated from sputum and bronchial fluid samples of active pulmonary tuberculous patients who were diagnosed and treated in Samsun Pulmonary Disease and Chest Surgery Hospital from 2005 to 2010. We have reproduced positive samples by Lowenstein- Jehnsen (LJ) culture examination in a microbiology laboratory; then the single and first positive isolates from each patient enrolled in the study were used.

Homogenisation and decontamination

The bronchioloalveolar lavage fluids (BAL) and sputum samples of suspected tuberculosis patients were subjected to homogenisation and decontamination process by N-Acetyl-L-Cistein (NALC) and sodium hydroxide (NaOH) mixture and then to LJ culture .

Culture and identification

The resistance characteristics related to the primary antituberculous drugs (SM, INH, RIF, ETM) of 1607 units of MTC strains which were produced in Solid (Lowenstein-Jensen) and liquid (BACTEC 12B and the Mycobacteria Growth IndicatorTube (MGIT)) nutrient media were detected by the BACTEC 960 (Becton Dickinson, USA) system. After the reproduction process, MTC and nontuberculous mycobacteria (MOTT) separation was made by a p-nitro-α-β-hydroxy-asetilamino-propiofen (NAP) test.

Susceptibility test against primary antituberculous drugs

We used BACTEC MGIT 960 System to determine the susceptibility of *M. tuberculosis* Complex isolates to major anti tuberculous agents. The susceptibility test was performed according to the manufacturer's recommendations (Becton, Dickinson and Company USA). This research is the result of several years study but we used same method (Trade mark; Becton, Dickinson and Company USA) and same drug concentrations in six years overall susceptibility tests. So there was not any change in the brand of drugs. Dilutions of drugs used in this study are shown in Table 1.

Quality control

For quality control of susceptibility tests, the ATCC 27294 (H37Rv) strain was used. The Rome Supranational Tuberculosis Reference Laboratory of World Health Organization and Turkey's Refik Saydam Hygiene Center Tuberculosis Research Laboratories, cooperated in the preparation of a quality control program which was implemented in this study.

Statistical analysis

We used SSPS.15 and minitab.16 programme for statistic analysis on INH and MDR ratios by years.

RESULTS AND DISCUSSION

We introduced the state of primary resistance in MTC to four major antituberculous agents in the last six years in Samsun province (the central city of Turkey's Middle Black sea region, with a population of 1.3 million) and we also retrospectively compared these values with the other regional and national determinants on drug resistance. In this study, 16,932 patient specimens were sent to our

Table 2. The primary resistance rates of *Mycobacterium tuberculosis* complex isolates according to years.

Type of resistance	2005	2006	2007	2008	2009	2010	Total (n)
Single drug resistance							
SM (%)	1(0.32)	5(1.67)	12(5)	7(2.99)	5(2.02)	13(4.69)	43(2.67)
INH (%)	27(8.64)	25(8.38)	32(13.33)	26(11.11)	21(8.46)	33(11.91)	164(10.20)
RIF (%)	1(0.32)	5(1.67)	6(2.50)	-	2(0.80)	2(0.72)	16(0.99)
ETM (%)	-	7(2.34)	7(2.91)	11(4.7)	2(0.80)	1(0.36)	28(1.74)
Any (total) monodrug resistance							
SM(%)	7(2.25)	14(4.70)	28(11.67)	14(5.98)	23(9.27)	24(8.66)	110(6.84)
INH(%)	49(15.80)	39(13.08)	58(24.17)	39(16.66)	41(16.53)	50(18.05)	276(17.17)
RIF(%)	19(6.12)	11(3.69)	20(8.33)	5(2.13)	15(6.04)	15(5.41)	85(5.28)
ETM(%)	5(1.61)	9(3.02)	20(8.33)	23(9.82)	4(1.61)	5(1.80)	66(4.10)
Resistance to 2 drugs							
INH-RIF(%)	13(4.19)	3(1.01)	5(2.08)	1(0.42)	4(1.61)	5(1.80)	31(1.93)
INH-SM(%)	3(0.96)	6(2.01)	5(2.08)	1(0.42)	7(2.82)	5(1.80)	27(1.68)
INH-ETM(%)	1(0.32)	2(0.67)	6(2.50)	5(2.13)	1(0.40)	0	15(0.93)
RIF-SM(%)	0	0	0	0	3(1.20)	0	3(0.18)
RIF-ETM(%)	0	0	0	1(0.42)	0	1(0.36)	2(0.12)
SM-ETM(%)	0	0	1(0.42)	0	0	0	1(0.06)
Resistance to 3 drugs							
INH-RIF-SM(%)	1(0.32)	3(1.01)	4(1.67)	0	5(2.02)	4(1.44)	17(1.05)
INH-RIF-ETM(%)	2(0.64)	0	0	0	0	1(0.36)	3(0.18)
INH-SM-ETM(%)	0	0	1(0.42)	3(1.28)	1(0.40)	0	5(0.31)
RIF-SM-ETM(%)	0	0	0	0	0	0	0
Resistance to 4 drugs							
INH-RIF-SM-ETM(%)	2(0.64)	0	5(2.08)	3(1.28)	0	2(0.72)	12(0.74)
MDR strains							
MDR(%)	21(6.77)	6(2.01)	14(5.83)	4(1.70)	9(3.62)	12(4.33)	63(3.92)
Total(n)	n:310	n:298	n:240	n:234	n:248	n:277	n:1607

microbiology laboratory. Only the single and first isolates of each new patient enrolled in the drug susceptibility study were used. The test was performed on a total of 1607 isolates from January 2005 to December 2010. Three hundred and eighty nine (24.20%) isolates were obtained from females and 1218 (75.80%) were obtained from male patients. All of the isolates were obtained from respiratory samples consisting of 113 (7.03%) bronchioloalveolar lavage (BAL) and 1494 (92.7%) sputum samples. 1240 (77.16%) of isolates showed sensitivity to all four drugs. The distribution of the samples studied over the years, and resistance profiles are presented completely in Table 2.

Because of global increasing in multidrug resistance rates we used statistical analysis on type of drug and years to see the changes in our province. Statistic results for INH and MDR ratios were analysed by SSPS.15 and minitab.16 programmes (Table 3). INH drug resistance rates between 2005-2007 differed a 5% significance level according to the dual rate comparison over the years. INH drug resistance rates differed between 2005-2008, 2006-2007 and 2007-2008, and these were statistically significant according to the dual rate comparison over the years. The relationship between INH drug sensitivity and years was determined (chi-square, p=0.025). MDR drug resistance rates differed between 2005-2008, 2006-2007 and 2007-2008 and these were statistically significant according to the dual rate comparison over the years. Between 2005 and 2006, MDR resistance rates differed at a 5% significance level according to the same method.

Due to previously predicted MDR-TB rates of 20% fort he present year(2011) in the world, resistance testing in all high risk cases for drug resistance is recommended by WHO (WHO/HTM/TB/2010,3). MDR-TB diagnosis and

Table 3. Statistical analysis of isoniazid and multidrug resistance of the study (with SSPS.15 and minitab.16).

Years	INH resistance		MDR resistance	
	Z account	P	Z account	P
2005-2006	0.94	0.348	2.34	0.019
2005-2007	-2.44	0.015	-0.25	0.806
2005-2008	-0.29	0.775	2.44	0.015
2005-2009	0.01	0.992	1.11	0.268
2005-2010	-0.76	0.447	0.69	0.491
2006-2007	-3.27	0.001	-2.27	0.023
2006-2008	-1.15	0.252	0.20	0.841
2006-2009	-0.87	0.383	-1.13	0.259
2006-2010	-1.66	0.097	-1.59	0.113
2007-2008	2.04	0.042	2.37	0.018
2007-2009	2.34	0.019	1.23	0.219
2007-2010	1.68	0.093	0.86	0.390
2008-2009	0.28	0.779	-1.26	0.207
2008-2010	-0.43	0.667	-1.71	0.088
2009-2010	-0.73	0.465	-0.42	0.676

treatment must be done according to international guidelines (WHO/HTM/TB/2010-3; WHO/HTM/STB/2010-2). The World Report 2008 on antituberculosis drug resistance' reported global risks for one or more drug resistance rates 0-56.3% in new cases, 0- 85.9% in patients treated previously, and 0-68.9% in all cases (WHO, 2009). According to the Turkish Ministry of Health's 2008 report, 18,452 tuberculous patients were recorded. The incidence of tuberculosis in Samsun province in 2008 was 25.8 and the case speed was 28.0 in Samsun. 414 new tuberculosis cases were diagnosed in 2005, 384 in 2006, 327 in 2007 and 346 in 2008 (Bozkurt et al., 2010).

According to the results in the Turkey (2008) TB report, a total of 4,963 drug susceptibility patient test were examined and 19.1% were found to be resistant to at least one drug. The highest rate of resistance among drugs belonged to isoniazid. Drug susceptibility tests detected the total multidrug resistant (MDR-TB) ratio to be 5.3% (263 people), 3% in new cases and 18.6% in cases having been previously treated (Table 4) (Bozkurt et al., 2010). We found 15.74% primary resistance to at least one drug in this study. Mono drug primary resistance rates for SM, INH, RIF, ETM were 6.84, 17.17, 5.28 and 4.10% respectively. INH primary resistance rates are higher than the other major drugs in the present study and much greater than Turkey's rates. MDR-TB has been identified as 3.92%. This rate is lower than the global estimated average ratio (20%) and Turkey's ratio (5.3%) but higher than national primaries (3.0%) (WHO/HTM/STB/2010-2; Bozkurt et al., 2010). Due to a low ratio of susceptibility testing in the area (4963 tests in 18452 cases 26.89% for 2008), these results for the country may not reflect the real resistance state in Turkey; however, this study reflects the real and nearly exact drug resistance rates of provincial tuberculosis. The limitation of our study is not having the secondary resistance rates at the same time.

In recent years, a variety of resistance test studies against major drugs have been performed in our country and the comparative results are shown in Table 4.

We have also analysed the rates of primary, secondary and total resistance in Turkey in Table 5, by using the 2005-2008 data of Turkish Ministry of Health (Bozkurt et al., 2010). Secondary resistance rates were extremely higher than primaries. The ratio of drug resistance to INH was high compared with the other major drugs. In addition, multiple and single drug resistances in these years were close to each other on some level in our country and in our study.

Regional differences in the resistance prevalence studies were published by authors. Arseven and his colleagues reported results from the provinces of the Eastern Black Sea region between the years 1985 and 1990. A total of 564 (40.6%) of the 1388 culture-positive TB patients were determined to be resistant to at least one of the following: INH, RIF, SM, and ETM. Drug resistance rates against INH, RIF, SM and ETM were 29.6, 17.1, 23.3 and 8.8% respectively. They measured the poly (more than one) drug-resistance rate to be 22.4%, and the rate of MDR strains to be 13% (Arseven et al., 1995). Saral and his colleagues found the rates of resistance to INH, RIF, SM and ETM in a study as 24.6, 15.8, 9.9 and 18.8% respectively. In the same study, the rate of MDR-MTC was reported as 14.7% (Saral et al., 2007). Aydın et al. (2011) performed a similar study in Trabzon and found the single drug resistance to INH to be 6.1%, RIF 0.5% , SM 5.2% and ETM 2.4%. In the

Table 4. Some study results about primary resistance in Turkey.

Reference	City/Region	Date	Number of isolates (susceptibility test studied)	Primary resistance rates of major anti-TB drugs			
				SM(%)	RIF(%)	INH(%)	EMB(%)
Turkey (2008)	Whole country	2005-2008	15735	8.37	6.77	13.37	4.23
Present study	Samsun	2005-2010	1607	6.84	5.28	17.17	4.10
Yolsal et al	Regional metaanalysis	1984-1989	368	8.8	5.7	14.4	2.2
Yolsal et al	Regional metaanalysis	1990-1995	2848	10.1	8.9	8.8	3.0
Doğan et al	Sivas	1999-2004	316	15.2	4.1	19.9	2.5
Güneri et al	Aegean region	1999-2001	387	0	5.7	12.4	0
Çetinkaya et al	Elazığ	1989-1994	125	16.6	7.6	11.5	1.2
Sürücüoğlu et al	Manisa	1997-2003	285	13.3	6.0	14.4	8.4
Talay et al. (2003)	İstanbul	1997-2000	135	13.3	3.0	8.8	2.2
Otkun et al (38)	Edirne	1996	44	32.0	11.0	30.0	9.0
Karadağ et al. (2004)	Samsun	2004	50	4.0	4.0	8.0	2.0
Aydın et al. (2011)	Zonguldak	2003-2005	99	13.1	2.0	18.2	3.0
Aydın et al. (2011)	Trabzon	2005-2010	212	13.7	5.7	17.5	5.7
Korkmaz et al. (2002)	Gaziantep	2002-2003	104	2.89	10.58	25	18.27
Korkmaz et al. (2002)	Gaziantep	2002-2003	104	2.89	10.58	25.04	25.96

same study, of the 212 isolates, 25 (11.8%) strains were multiple drug resistant 10(4,7%) (Aydın et al., 2011). Many studies have been performed on relevant city analysis of TB for mono drug and multidrug resistance (Şenol et al., 2004; Uçar et al., 2010). For example Aydın et al. (2011) analysed 125 strains in the province of Zonguldak and reported the sensitivity rate to all drugs as 69.6%, resistance rate to INH as 23.2% and the rate of multidrug resistance as 8% in 2005 (Aydın et al., 2008). In our country, it has been reported that INH+RIF resistant strains are 2.7% in Isparta, 7 and 3% (two studies done at different times) in Edirne, and 12.8 and 19.6% in Gaziantep (two different studies) (Yaylı et al., 2003; Tansel et al., 2003; Balcı et al., 1999; Gani et al., 2002). Another study reported resistance rates in our city (Samsun) as 8% to INH, 4% to RIF, 4% to SM, 4% to ETM, 2% to pyrazinamide and 4% to MDR-TB strains in 2004 (Karadağ et al., 2004).

The prevalence of MTC drug resistance varies from one part of the world to another (Jaffar et al., 2005). In the United States, drug-resistant tuberculosis was detected in 14.2% in 1991 and 10% in 1997 (Bloch et al., 1994; Espinal et al., 2001). In the United States, isoniazid resistance was the most prevalant and accounted for 8%. Isoniazid resistance has ranged from 0% in New Caledonia to 7.9% in Mozambique, and was 10% in India (Pereira et al., 2005). An article from Saudi Arabia reported the fact that the rate of resistance to isoniazid varied from one part of the country to another such as: 4.2-7.2% in Riyadh, 6% in Dammam and Taif, 10.3- 28.7% in Jeddah and 41% in Gizan. A 15-year study found rates of resistance to INH in different drug concentrations as follows: 12.5% resistance in INH (1 µg/mL), and 2.9% resistance in INH (5 µg/mL) in Dhahran (Jaffar et al., 2005). The prevalence of MDR-TB among new TB cases may differ in diverse geographies such as 14% in Estonia, 9% in China, Henan province, 9% in Lithuania, 9% in Russia, Ivanovo province, 5% in Iran and 4.5% in China, Zhejiang province (Espinal et al., 2001). In the early stages of tuberculosis treatment, the most potent bactericidal drug is isoniazid. INH resistance may also be an indicator of success in the treatment of tuberculosis. Storla and colleagues reported a high treatment failure in areas with high resistance to INH in Bangladesh (Storla et al., 2007).

Directly observed therapy (DOT) in TB patients has been successfully completed in some countries such as the United States and a significant fall in TB and MDR-TB cases was provided contrary to the global rising in the number of

Table 5. The rates of primary, secondary and total drug resistance types in Turkey in 2005-2008.

Drugs	Resistance type	2005 (%)	2006 (%)	2007 (%)	2008 (%)
INH	Primary	9.0	10.7	11.9	11.3
	secondary	27.4	23.8	27.6	27.9
	Total	11.5	12.6	14.4	13.8
RIF	Primary	4.4	4.5	4.9	3.9
	Secondary	21.1	19.8	18.7	21.8
	Total	6.7	6.7	7.1	6.6
ETM	Primary	3.0	3.6	2.8	3.4
	Secondary	10.0	13.2	8.3	9.6
	Total	4.0	5.0	3.6	4.3
SM	Primary	7.0	8.4	7.1	6.5
	Secondary	15.2	17.0	13.8	12.9
	Total	8.1	9.7	8.2	7.5
MDR-TB	Primary	3.1	3.2	2.9	3.0
	Secondary	17.7	16.6	15.5	18.6
	Total	5.1	5.1	4.9	5.3

multidrug-resistant tuberculosis. WHO declared the fact that between 1995 and 2009, a total of 41 millionTB patients were successfully treated in DOT, and up to six million lives were saved, including two million women and children.

In conclusion, resistance to INH and RIF continues, almost in a straight line in Samsun Province. DOT must not be disregarded due to global rises in drug resistant MTC strains. Surveillance of the primary resistance patterns is important in assessing the quality of chemotherapy programs over several years.

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