Integration of *Mycobacterium tuberculosis* Drug Susceptibility Testing and Genotyping with Epidemiological Data Analysis To Gain Insight into the Epidemiology of Drug-Resistant Tuberculosis in Malatya, Turkey

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Drug-resistant tuberculosis (TB) presents a major challenge to global TB control. To gain a better understanding of drug-resistant TB epidemiology in Malatya, Turkey, we conducted the present study using 397 *Mycobacterium tuberculosis* clinical isolates collected from Malatya, Turkey, in recent years (2000–2007). Resistance to any anti-TB drug was found in 29% (114 of 397) of the study isolates, while the multidrug resistance (MDR) rate was ~4.5% (18 of 397). Resistances to isoniazid (15.5%) and streptomycin (13.4%) were about twice as high as resistance to rifampin (RMP) (6.3%) and ethambutol (EMB) (6.0%). Importantly, 28% (7 of 25) of the RMP-resistant isolates were non-MDR isolates, as when a significant proportion of RMP-resistant isolates in a population are non-MDR, the predictive value of molecular detection of RMP resistance for MDR can be significantly reduced. Both identical and varied drug resistance patterns were seen in the same genotyping-defined clusters, suggesting that both primary and acquired resistance have contributed to the drug-resistant TB epidemic in Malatya, Turkey. In addition, drug-resistant cases were found to be more likely to be males (odds ratio [95% confidence interval], 1.82 [1.13, 2.94]), suggesting a potential role of gender in the epidemiology of drug-resistant TB in the study population. This study demonstrates that the integration of drug susceptibility testing with genotyping and epidemiological data analysis represents a useful approach to studying the epidemiology of drug-resistant TB.

Tuberculosis (TB) remains an important global public health problem, and global TB control is further challenged by the rising epidemics of drug-resistant TB worldwide (29). In 2008, the World Health Organization (WHO) reported that worldwide resistance to any of the anti-TB drugs accounted for 20% of all reported TB cases while an estimated 5.3% of all reported TB cases had multidrug resistance (MDR), defined as resistance to, at least, isoniazid (INH) and rifampin (RMP) (28). Furthermore, globally, only 10% of the roughly 500,000 people who develop MDR TB each year receive treatment, leading to more possible cases of MDR TB (9). Because of the disparities in TB control around the world, a better understanding of the dynamics and driving forces of drug-resistant TB epidemics would contribute to the development of more effective strategies for global TB control.

Turkey, with a population of around 70 million, had an annual TB incidence of 27.9 per 100,000 people in 2007 (6). Although the TB incidence in Turkey has decreased by half since 1985, several studies have shown the proportion of drug-resistant TB cases to be higher than the global average (3, 5, 10, 11, 20, 24, 26). In 2005, Surucuoglu and colleagues reported that the rate of resistance to any anti-TB drugs among 355 isolates of *Mycobacterium tuberculosis* obtained from western Turkey was 21.1%, while another study found that among the 1,513 TB cases diagnosed in Istanbul, Turkey, in 2005, 19% were resistant to at least one drug (20, 25). These rates are similar to the rates of drug resistance found in some of the Eastern European countries that are considered to have the highest drug resistance rates in the world (3). The high drug resistance rate poses a major challenge to the control of TB in Turkey. Previous studies of different populations have found that both host and microbial factors can play a role in drug-resistant TB epidemics. Microbial factors such as specific spoligotype families have been implicated as risk factors. TB genotypes belonging to the Beijing and Latin American and Mediterranean (LAM) families are associated with drug resistance (18, 23).

Although previous studies reported high rates of drug-resistant TB infection in different regions of Turkey and several studies reported genotyping results of *M. tuberculosis* isolates collected from different regions of Turkey (10–12), few assessed associations of microbial and host characteristics with drug-resistant TB cases and attempted to determine the factors driving the epidemic of drug-resistant *M. tuberculosis* infection in the population. To gain a better understanding of the epidemiology of drug-resistant TB in Malatya, Turkey, we analyzed 397 *M. tuberculosis* clinical isolates collected from Malatya, Turkey, during the time period between 1 January 2000 and 31 December 2007 and their corresponding epidemiological data. Malatya is the third biggest city in Turkey and has a population of approximately 722,000 and an annual TB incidence of 28.5 per 100,000 (6).
MATERIALS AND METHODS

Study sample and patient data. The study sample included 397 TB cases whose diagnoses were confirmed by mycobacterial culture performed at Turgut Ozal Medical Center, Inonu University, Malatya, Turkey, during the time period from 1 January 2000 to 31 December 2007. One isolate was obtained from each patient. The study sample represented all the cases diagnosed in the study period for which a live culture of M. tuberculosis isolate and clinical and laboratory data were available. There were approximately 80 to 90 culture-positive cases that were diagnosed each year in Malatya during the study period, and each year, two or three cases were identified as MDR (11). Thus, our study sample is estimated to represent approximately 58% of the culture-positive TB cases in Malatya during the study period. The demographic, clinical, and laboratory data of the patients were retrieved from the existing database established by the Turkish authors of this report. This study was approved by the University of Michigan Institutional Review Board and the Inonu University Ethical Committee.

Isolation and drug susceptibility testing of the study isolates. The isolation and drug susceptibility testing of the study isolates were done at the Clinical Microbiology Laboratory, Turgut Ozal Medical Center, Inonu University, Malatya, Turkey. This laboratory is a member of the External Quality Control Program of the National TB Reference Laboratory at Refik Saydam National Public Health Agency in Ankara, Turkey. The isolates were identified as M. tuberculosis based on the results of nitrate reduction and niacin accumulation tests, the BACTEC M. tuberculosis-p-nitro-o-acylamino-β-hydroxypropiophenone test (Becton Dickinson, Sparks, MD), and the growth characteristics. All the study isolates were tested for susceptibility to the four anti-TB drugs routinely used in Turkey, including INH, RMP, streptomycin (SM), and ethambutol (EMB). Drug susceptibility testing was done by the modified 1% proportion method using the BACTEC 460 radiometric system or the MGIT 9600 system (Becton Dickinson, Sparks, MD) (11). Drug resistance was defined as greater than 1% growth in the presence of 0.1 μg/ml INH, 2 μg/ml RMP, 2 μg/ml SM, or 2.5 μg/ml EMB. MDR was defined as resistance to at least INH and RMP (11).

Genotypic characterization. To gain a better understanding of the characteristics of M. tuberculosis isolates causing drug-resistant TB in Malatya, Turkey, we compared the distribution of the major spoligotyping-defined genetic families among the drug-resistant isolates with that among the drug-susceptible isolates. The spoligotypes of the study isolates were obtained using standard spoligotyping methods described previously (11, 19). Briefly, the direct repeat locus of M. tuberculosis strains was amplified with Dra and Drb primers, with primer Dra biotinylated at the 5’ end. The amplified products were hybridized to a set of 43 oligonucleotides, and hybridized DNA was detected by chemiluminescence. The spoligotype of each isolate was compared to the international spoligotyping database of the Pasteur Institute of Guadeloupe and defined as belonging to one of the major phylogenetic clades, according to signatures provided in SpolDB4 (7).

In addition, in order to assess the relative contributions of primary resistance due to the transmission of drug-resistant isolates and acquired resistance due to treatment default and failure to the burden of drug resistance, we examined the number of drug resistance patterns observed within each cluster of isolates defined based on a combination of IS6110 and spoligotyping results that were obtained previously by one of the authors of the present report using commonly used standards (4, 11). Clustered isolates were defined as any two or more isolates having identical IS6110 fingerprinting patterns comprising more than five bands, having identical IS6110 fingerprinting patterns comprising five or fewer bands and identical spoligotyping patterns, or having similar (plus, minus, and shift one band) IS6110 patterns comprising more than five bands and identical spoligotypes. The remaining isolates were defined as unique isolates. Although our convenient, non-population-based sample was not expected to allow for an accurate estimation of clustering rate for either group in comparison, we believe that the missing cases were randomly distributed in each of the comparative groups. Thus, the comparison should be valid.

Statistical analysis. To gain a better understanding of the microbial and host characteristics associated with drug-resistant TB, we compared the distributions of the demographic, microbial, and clinical characteristics, including age, sex, and disease sites, between drug-resistant and -susceptible groups by χ2 test. Furthermore, we compared the distributions of all the available demographic, microbial, and clinical characteristics between MDR and non-MDR resistant groups as well as between MDR and drug-susceptible groups by χ2 test, or Fisher’s exact test, as appropriate. The magnitudes of the associations of the analyzed characteristics with any drug resistance were measured by odds ratios and 95% confidence intervals. Multivariate logistic regression was performed to identify independent risk factors for having any type of drug-resistant TB, controlling for potential confounders. All of the statistical analyses were done using SAS, version 9.0 (SAS Institute, Cary, NC).

RESULTS

Frequency of drug resistance and MDR. Of the 397 study isolates, 114 (28.7%) were resistant to at least one of the four first-line anti-TB drugs tested and 18 (4.5%) were MDR, accounting for 15.8% of the total number of the drug-resistant isolates. The proportion of isolates resistant to each of the four tested anti-TB drugs varied. Resistances to INH (15.5%, 62 of 397) and SM (13.4%, 53 of 397) were almost twice as high as resistances to RMP (6.3%, 25 of 397) and EMB (6.0%, 24 of 397), respectively. Among the 114 resistant isolates, 12 different drug-resistant patterns were observed (Fig. 1). Of the 25 RMP-resistant isolates found in our study sample, 18 (72%) were defined as MDR and the remaining 7 (28%) were not resistant to INH and therefore defined as non-MDR.

Host characteristics. Of the 397 study subjects, 238 (60%) were male and 159 (40%) were female. A significantly higher proportion (69.3% versus 56.2%, P = 0.02) of males was found among drug-resistant cases than among drug-susceptible cases (Table 1).

The age of the study patients ranged from less than 1 year old to 89 years old, with a mean of 33.9 years and a median of 32 years. The age composition of the study patients was as follows: ≤15 years, 12.8% (n = 51); 16 to 30 years, 28.2% (n = 112); 31 to 45 years, 21.9% (n = 87); 46 to 60 years, 15.1% (n = 60); and >60 years, 7.3% (n = 29). The median ages for the drug-resistant and drug-susceptible groups were 31.5 and 32.0 years old, respectively. The age distribution did not differ statistically significantly between the resistant and susceptible groups (Table 1). However, the age distribution was statistically significantly different between the MDR group and the drug-susceptible group (P = 0.02). A significantly higher proportion of patients whose age was between 16 and 45 years was found among MDR cases than among drug-susceptible cases (Table 1). In contrast, a significantly higher proportion of patients from the remaining age groups (excluding the unknown group) was found among the drug-susceptible cases than among the MDR cases (36.8% versus 5.6%, P = 0.02).

The primary disease site distributions for drug-resistant and drug-susceptible groups were similar (Table 1). The distributions of the host factors were similar between MDR isolates and non-MDR isolates with drug resistances that do not define them as MDR (data not shown). We also compared the host characteristic distributions for the INH-resistance-alone and drug-susceptible groups. The findings are similar to those from the comparison between the group with resistance to any drugs and the susceptible group.

Microbial characteristics. (i) Spoligotypes and drug resistance. A total of 10 spoligotype superfamilies were found among the 397 isolates. The ill-defined T superfamily was the most common spoligofamily found in the study sample, representing 46.3% of the study isolates. However, the ill-defined T superfamily was not found to be more prevalent among the drug-resistant cases than among the drug-susceptible cases (Table 1). Although the overall distributions of the different spoligotype superfamilies were not statistically significant different between the resistant and the susceptible groups, the Latin American and Mediterranean (LAM) superfamily isolates and the spoligofamily-undetermined isolates appeared to be overrepresented in the drug-resistant group whereas the
Haarlem (H) family isolates were remarkably underrepresented in the drug-resistant group (Table 1), compared to their distributions in the drug-susceptible group. There was no significant differences between the distributions of spoligotype superfamilies for MDR and drug-susceptible groups (Table 1) or for MDR isolates and non-MDR isolates with drug resistances that do not define them as MDR (data not shown).

(ii) Clustering and drug resistance.

Of the 397 study isolates, 132 (33.2%) were determined to belong to one of 44 clusters identified. Of these 44 clusters, 18 (40.9%) contained exclusively drug-susceptible isolates and the remaining 26 (59.1%) contained both resistant and susceptible isolates. There was no cluster that included exclusively drug-resistant isolates. Intracluster drug resistance pattern variations were observed in 5 (19.2%) of the 26 clusters containing resistant isolates. In the remaining 21 clusters, only one resistance pattern was observed within each cluster. Of the five clusters that showed more than one resistance pattern within the same cluster, one contained multiple isolates sharing the same resistance pattern, in addition to isolates having different patterns. The proportion of drug-resistant isolates in each of the 26 clusters containing both drug-resistant and susceptible isolates ranged from 20% to 67%. Of these 26 clusters, the largest contained 17 cases, of which 5 were resistant to all the four drugs. The clustering rate observed among the resistant isolates or among the MDR isolates was similar to that observed among the susceptible isolates (Table 1). However, it is worth noting that two isolates with identical MDR patterns were found in two clusters, respectively.

(iii) Risk factors of drug resistance.

Being of the male sex was statistically significantly associated with drug resistance.

TABLE 1. Distribution of host and microbial characteristics among drug-susceptible, drug-resistant, and MDR TB cases in Malatya, Turkey, 2000–2007

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Pan-sensitivity</th>
<th>Any resistance</th>
<th>Multidrug resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. (%) of cases</td>
<td>No. (%) of cases</td>
<td>P value*</td>
</tr>
<tr>
<td>Age (yr)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–15</td>
<td>37 (13.07)</td>
<td>14 (12.28)</td>
<td>0.15</td>
</tr>
<tr>
<td>16–30</td>
<td>76 (26.86)</td>
<td>36 (31.38)</td>
<td>0.00</td>
</tr>
<tr>
<td>31–45</td>
<td>55 (19.43)</td>
<td>32 (28.07)</td>
<td>0</td>
</tr>
<tr>
<td>46–60</td>
<td>44 (15.55)</td>
<td>16 (14.04)</td>
<td>1</td>
</tr>
<tr>
<td>≥61</td>
<td>23 (8.13)</td>
<td>6 (5.26)</td>
<td>0 (0.00)</td>
</tr>
<tr>
<td>Unknown</td>
<td>48 (16.96)</td>
<td>10 (8.77)</td>
<td>1 (5.60)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>159 (56.18)</td>
<td>79 (69.30)</td>
<td>0.02</td>
</tr>
<tr>
<td>Female</td>
<td>124 (43.82)</td>
<td>35 (30.70)</td>
<td>0</td>
</tr>
<tr>
<td>Primary disease site</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulmonary</td>
<td>206 (72.79)</td>
<td>88 (77.19)</td>
<td>0.38</td>
</tr>
<tr>
<td>Extrapulmonary</td>
<td>77 (27.21)</td>
<td>26 (22.81)</td>
<td>1</td>
</tr>
<tr>
<td>Spoligotypes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>superfamily</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>136 (48.06)</td>
<td>48 (42.11)</td>
<td>0.20</td>
</tr>
<tr>
<td>LAM</td>
<td>50 (17.67)</td>
<td>27 (23.68)</td>
<td>0</td>
</tr>
<tr>
<td>H</td>
<td>45 (15.90)</td>
<td>11 (9.65)</td>
<td>1</td>
</tr>
<tr>
<td>Undetermined</td>
<td>34 (12.01)</td>
<td>19 (16.67)</td>
<td>4</td>
</tr>
<tr>
<td>Other*</td>
<td>18 (6.36)</td>
<td>9 (7.89)</td>
<td>2</td>
</tr>
<tr>
<td>Clustered</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>93 (32.86)</td>
<td>39 (34.21)</td>
<td>0.80</td>
</tr>
<tr>
<td>No</td>
<td>190 (67.14)</td>
<td>75 (65.79)</td>
<td>11</td>
</tr>
</tbody>
</table>

* P values (relative to the values for the drug susceptible isolates) obtained from Pearson’s χ² tests unless otherwise noted.

a Other superfamilies included Beijing (n = 4), Central Asian Family1/Delhi (n = 1), Manu (n = 1), S (n = 8), U (n = 8), and X (n = 4).

P value obtained from Fisher’s exact test.
(adjusted odds ratio [95% confidence interval], 1.82 [1.13, 2.94]). All the other variables analyzed, including age, spoligotype family, and DNA fingerprinting clustering, and disease site were not statistically significantly associated with drug-resistant TB. None of the microbial characteristics investigated in this study was statistically significantly associated with anti-TB-drug resistance. The small sample size of MDR TB cases in our study did not allow us to assess the risk factors for MDR TB in this study.

**DISCUSSION**

This study is the first drug-resistant TB epidemiology study conducted in Malatya, Turkey, via an integrated approach combining M. tuberculosis drug susceptibility testing, genotyping, and epidemiological data analysis. A high percentage (28.7%) of the study isolates were resistant to one or more anti-TB drugs, with the resistance to INH and SM being approximately twice the resistance to RMP and EMB. MDR isolates accounted for 4.5% of the study sample. As many as 28% of RMP-resistant isolates were non-MDR isolates. Neither spoligotype families nor clustering of M. tuberculosis isolates was statistically significantly associated with drug resistance. However, males had a significantly elevated risk for having drug resistance compared with females (adjusted odds ratio [95% confidence interval], 1.82 [1.13, 2.94]). The rate of resistance to any drug (28.7%) found in this study is 7.6 to 9.7% higher than previously reported in Turkey and approximately 9% higher than the WHO 2008 global estimate (20, 25, 26, 28). The MDR rate (4.5%) found in the current study is slightly lower than the estimated 5.3% global prevalence (28). These data suggest the need for a better understanding of the factors contributing to the epidemics of drug-resistant TB in Malatya, Turkey.

It is well known that drug-resistant TB in a population can be the result of the spread of drug-resistant strains of M. tuberculosis (primary drug resistance), the development of drug resistance in the drug-susceptible M. tuberculosis strain-infected individuals due to treatment default or failure (acquired resistance), or both (28). In our study, due to the lack of information on the study subjects’ history of TB and TB treatment, we were unable to define each case as primary resistance versus acquired resistance. However, two findings from the study suggest that both primary and acquired resistances have contributed to the high burden of drug-resistant TB in the study population. First, all the clusters that contained drug-resistant isolates showed more than one drug resistance patterns. Second, while some isolates in the same clusters shared identical drug resistance patterns, others had unique drug resistance patterns.

While our non-population-based sample has inevitably led to the underestimation of the TB clustering rate in Malatya, Turkey, one-third of the study isolates were identified as clustered isolates, indicating the high degree of active transmission of TB in Malatya, Turkey, during the study period. Our study serves as an example of how the integration of drug susceptibility testing and genotyping of M. tuberculosis clinical isolates can provide insight into the dynamic of drug-resistant TB epidemiology in developing countries where comprehensive patient data, especially information on TB treatment history, are often unavailable.

The likelihood that acquired drug resistance has contributed to the high burden of drug-resistant TB in Malatya is also supported by several facts regarding the inadequate TB control program in Turkey. In Turkey, TB control is mainly done by TB dispensaries that are scattered across the country, university hospitals, and chest disease hospitals. However, due to the lack of culture facilities in the TB dispensaries, a considerable proportion of TB patients in Turkey are usually treated without drug susceptibility testing. Moreover, the nationwide implementation of directly observed therapy of TB did not begin until 2008.

The high frequency of the INH and SM resistance found in the study sample is consistent with the national data on INH and SM resistance in Turkey (6). In Turkey, INH, SM, and RMP are among the main drugs used for TB treatment, while EMB is used occasionally. Since the rate of spontaneous mutation related to RMP is lower than those related to INH and SM, the observed higher rates of INH and SM resistance in Turkey are within the expectation (30).

The finding that as many as 28% of the RMP-resistant isolates were not MDR is similar to the data reported in the 2009 Tuberculosis Control Report of Turkey. According to the report, between 2005 and 2007, approximately 30% of RMP-resistant isolates in Turkey were non-MDR isolates (6). This finding has an important implication for global MDR TB diagnosis because RMP resistance has been used as a marker for molecular detection of MDR TB (1, 22). Since different populations may have had different levels of access to different anti-TB drugs over time, the relative frequency of M. tuberculosis isolates resistant to a given anti-TB drug may vary from population to population. When a significant proportion of RMP-resistant isolates in a population are non-MDR, the predictive value of RMP resistance for MDR TB is significantly reduced. It is therefore important to determine the frequency of MDR M. tuberculosis isolates among RMP-resistant isolates in different geographic regions.

Spoligotyping is a method by which M. tuberculosis isolates can be assigned into different phylogenetic families. Some previous studies found no association between spoligotype superfamilies and drug resistance (2, 27), and some showed that the Beijing, LAM, and Haarlem families were associated with drug-resistant TB (13, 18). Durmaz et al. reported that the T, LAM, and H superfamilies were the most common spoligotype superfamilies found among 76.5% of the drug-resistant isolates collected from four different regions of Turkey (12). In the present study, we attempted to assess the association between spoligotype superfamily and drug resistance of M. tuberculosis. The LAM superfamily seemed to be the most likely to be associated with drug resistance, while the H superfamily appeared to be the least likely to be associated with drug resistance. However, none of the associations were statistically significant. While the failure to find a statistically significant association between genetic families of M. tuberculosis and drug resistance in the previous and current studies may indicate a true absence of the associations, it could also be due to the insufficient statistical power of the small sample sizes used in all of these studies, including the current study. Large-scale studies using population-based samples would allow a more
accurate assessment of the relationship between spoligotype superfamilies and drug resistance of *M. tuberculosis* clinical isolates circulating in the study population.

There have been mixed findings regarding gender as a risk factor for having drug-resistant TB (14–16, 21). In the present study, while no association was found between drug resistance and age, a statistically significant association was found between male sex and drug resistance. However, the lack of comprehensive host data in the present study did not allow us to control for all potential confounders in the association analysis. The observed association, therefore, remains to be confirmed by future studies based on more comprehensive data on host characteristics.

The small sample size of MDR cases found in our study sample limited our ability to investigate the risk factors for having MDR TB. However, the identification of clusters containing more than one MDR isolate with identical resistance patterns suggests the important role of TB transmission in MDR TB epidemics in Malatya. This hypothesis was also supported by the finding that the majority of MDR cases were in the two age groups overrepresented among clustered cases.

In conclusion, drug-resistant TB is an important public health problem in Malatya, Turkey. Effective control of the epidemic of drug-resistant TB in the study population must aim at reducing both TB transmission and the occurrence of acquired resistance. This study demonstrates that the integration of drug susceptibility testing with genotyping and epidemiological data analysis represents a useful approach to studying the epidemiology of drug-resistant TB.

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16. Reference deleted.


