

Second-Line Drug Susceptibilities of Multidrug-Resistant *Mycobacterium tuberculosis* Isolates in Aegean Region - Turkey*

Nuri ÖZKÜTÜK¹
Süheyla SÜRÜCÜOĞLU¹
Hörü GAZİ¹
Meral COŞKUN²
Aydan ÖZKÜTÜK³
Beril ÖZBAKKALOĞLU¹

Aim: The emergence of multidrug-resistant tuberculosis (MDR-TB) is increasing, and the standard short-course regimen used for the treatment of TB is likely to be ineffective against MDR-TB, leading to the need for second-line drugs. In such situations, drug susceptibility testing is necessary to select an appropriate treatment regimen. Unfortunately, there are few studies showing the pattern of the second-line drug resistance in Turkey. We aimed to analyze the resistance to second-line anti-tuberculosis drugs of MDR strains of *Mycobacterium tuberculosis* isolated from the Aegean region of Turkey.

Materials and Methods: In this study, drug susceptibility testing of 40 MDR-TB strains isolated from the Aegean region of Turkey was performed using the BACTEC 460 TB radiometric system. Capreomycin, ethionamide, kanamycin, amikacin, clofazimine and ofloxacin were tested in 1.25 µg/ml, 1.25 µg/ml, 5.0 µg/ml, 1.0 µg/ml, 0.5 µg/ml, and 2.0 µg/ml concentrations, respectively.

Results: The results showed that 37.5% of the strains were resistant to ethionamide, 25% to capreomycin, 5% to kanamycin, amikacin and ofloxacin, and 2.5% to clofazimine. One (2.5%) of the 40 MDR-TB cases was defined as extensively drug-resistant tuberculosis (XDR-TB).

Conclusions: The results of the study indicate that the high rates of resistance to ethionamide and capreomycin may be a problem in the treatment of patients with MDR-TB; XDR-TB is not yet a serious problem in our region.

Key Words: *Mycobacterium tuberculosis*, multidrug-resistant tuberculosis (MDR-TB), second-line drugs, susceptibility tests, extensively drug-resistant tuberculosis (XDR-TB)

¹ Department of Microbiology and Clinical Microbiology, Faculty of Medicine, Celal Bayar University, Manisa - TURKEY

² Department of Microbiology and Clinical Microbiology, Chest Diseases and Chest Surgery Training and Research Hospital, İzmir - TURKEY

³ Department of Microbiology and Clinical Microbiology, Faculty of Medicine, Dokuz Eylül University, İzmir - TURKEY

Ege Bölgesinde Çok İlaça Dirençli *Mycobacterium tuberculosis* İzolatlarının İkinci Kuşak Antitüberküloz İlaçlara Duyarlılıkları

Amaç: Çok ilaca dirençli tüberküloz (MDR-TB) olgularındaki artış ve standart tüberküloz tedavi rejiminin MDR-TB olgularının tedavisinde yetersiz kalması ikinci kuşak anti tüberküloz ilaçların gereksinimine neden olmaktadır. Bu durumda doğru tedavi rejimine karar vermek için ilaç duyarlılık testleri gereklidir. Fakat ikinci kuşak anti tüberküloz ilaçlarının Türkiye'deki direnç paternini gösteren çok az çalışma vardır. Biz Ege bölgesinden izole edilen çok ilaca dirençli *Mycobacterium tuberculosis* suşlarının ikinci kuşak antitüberküloz ilaçlarına direncini belirlemeyi amaçladık.

Yöntem ve Gereç: Bu çalışmada, BACTEC 460TB radyometrik sistem kullanılarak, Türkiye'nin Ege bölgesinden soyutlanmış 40 MDR-TB suşunun ilaç duyarlılık testi yapıldı. Kapreomisin, etyonamid, kanamisin, amikasin, klofazimin ve ofloksasin sırasıyla 1.25 µg/ml, 1.25 µg/ml, 5.0 µg/ml, 1.0 µg/ml, 0.5 µg/ml ve 2.0 µg/ml konsantrasyonlarında test edildi.

Bulgular: Sonuçlar 40 MDR-TB olgusunun etyonamide % 37.5, kapreomisin %25, kanamisin, amikasin ve ofloksasine % 5 ve klofazimine ise % 2.5'inin dirençli olduğunu gösterdi. 40 MDR-TB olgusunun biri genişlemiş ilaç dirençli tüberküloz (extensively drug-resistant tuberculosis: XDR-TB) olarak tanımlandı.

Sonuç: Bölgemizde etyonamide ve kapreomisine direnç oranlarının yüksekliğinin MDR-TB'li hastaların tedavisinde sorun olabileceği ve XDR-TB'nin ise bölümümüzde şimdilik ciddi bir problem olmadığı görülmüştür.

Anahtar Sözcükler: *Mycobacterium tuberculosis*, çok ilaca dirençli tüberküloz (MDR-TB), ikinci kuşak anti tüberküloz ilaçlar, duyarlılık testleri, extensively drug-resistant tuberculosis (XDR-TB).

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Correspondence

Nuri ÖZKÜTÜK
Department of Microbiology and
Clinical Microbiology,
Faculty of Medicine,
Celal Bayar University,
45030 Manisa - TURKEY

ozkutuk@gmail.com

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Introduction

Although tremendous efforts to control tuberculosis (TB) have been undertaken at global and national levels, almost 2 million patients still die every year. Multidrug-resistant (MDR)-TB, defined as resistance to at least isoniazid and rifampin, has emerged as a worldwide threat to TB control (1). MDR-TB treatment requires the use of second-line drugs (SLDs) that are less effective, more toxic, and costlier than first-line isoniazid and rifampin (2). With the recent global resurgence of TB and concomitant rise in MDR strains of *Mycobacterium tuberculosis*, there is an increasing demand for determining the *in-vitro* susceptibilities of clinical isolates to antimicrobial agents other than the primary drugs (3). It was recommended that whenever an isolate of *M. tuberculosis* is resistant to rifampin or any two of the primary drugs, all SLDs should also be tested (4). Treatment of patients with MDR-TB with second-line anti-tuberculosis drugs should preferably be based on the results of susceptibility testing. Accurate and reliable laboratory drug susceptibility testing (DST) data to SLDs are the basic requirement of the directly observed treatment short course (DOTS)-Plus strategy, as they will support clinical decision-making and help to prevent the emergence of further drug resistance in patients with MDR-TB (5,6). Furthermore, these data are needed not only for individual case management, but also for drug resistance surveillance (7,8). Susceptibility testing of *M. tuberculosis* to SLDs is difficult, expensive, and not well standardized. An alternative is, therefore, to perform susceptibility testing of a sample of MDR strains in a region and to base the treatment of MDR-TB on these results (6).

Most mycobacteriology laboratories do not perform DST for SLDs and few studies are available showing the pattern of the SLD resistance in Turkey. The aim of this study was to analyze the resistance to second-line anti-tuberculosis drugs of MDR strains of *M. tuberculosis* isolated from the Aegean region of Turkey.

Materials and Methods

Strains

A total of 40 MDR *M. tuberculosis* isolates selected from our strain collection were included in this study. All of them had been isolated from individual patients in the Aegean region of Turkey, between 01.01.2004 – 31.06.2007. All clinical strains had been identified initially as *M. tuberculosis* using the NAP and standard microbiological tests (niacin accumulation and nitrate

reduction tests). Following identification, they had been tested for susceptibility to the first-line anti-tuberculosis drugs ethambutol, isoniazid, rifampin, and streptomycin using the BACTEC 460 TB system (Becton Dickinson, Sparks, MD, USA) by following the standard procedures and as recommended by the manufacturer (9). The strains had been stored at -70 °C, and were freshly subcultured on Lowenstein-Jensen (LJ) medium before use. *M. tuberculosis* H37Rv (ATCC 27294; susceptible to all drugs tested) was used as a strain for internal quality control.

Antimicrobial Agents

The drugs used were amikacin (AMI), capreomycin (CAP), kanamycin (KM), ofloxacin (OFX), ethionamide (ETH), and clofazimine (CFZ). All test drugs were obtained in a chemically pure form (all from Sigma, Taufkirchen, Germany, except for CFZ [Novartis International Pharmaceutical Ltd., Ringaskiddy Co. Cork, Ireland]).

AMI, CAP, and KM were dissolved in sterile distilled water; OFX was dissolved in a 0.1 N NaOH solution. Subsequent dilutions of these drugs were made in sterile distilled water. ETH and CFZ were dissolved in dimethyl sulfoxide and stored in the dark at room temperature. Subsequent dilutions were made in the same solvent. Stock solutions of each drug were made at 10,000 mg/ml concentration. Except for CFZ and ETH, which are considered self-sterilizing, all stock solutions were filtered in a sterile manner with a 0.22- μ m-pore-size polycarbonate filter, and the first 20% of the initial filtrate was discarded. All stock solutions except CFZ were stored at -70 °C in small aliquots. Frozen drug solutions were thawed once and then discarded. CFZ was prepared daily for each working batch.

Drug Susceptibility Testing (DST)

The DSTs were performed by the BACTEC 460 TB radiometric method (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD, USA) following the standard protocol (3,9). The final drug concentrations used were 1.0 μ g/ml for AMI; 1.25 μ g/ml for CAP; 5.0 μ g/ml for KM; 2.0 μ g/ml for OFX; 1.25 μ g/ml for ETH; and 0.5 μ g/ml for CFZ. Antimicrobials were always added in 0.1 ml quantities to a BACTEC 12B vial to achieve the desired concentrations. Susceptibility and resistance were judged by comparison of the change in the growth index of the control with that of the test drug as recommended. This interpretation gave susceptibility results at the 1% proportion basis.

Results

A total of 40 MDR *M. tuberculosis* strains isolated from patients with TB in the Aegean region of Turkey were tested for susceptibility to the second-line anti-tuberculosis drugs AMI, CAP, KM, OFX, ETH and CFZ. Susceptibility patterns identified are presented in the Table. Eighteen of the 44 (45%) strains were susceptible to all SLDs tested. Twenty-two (55%) strains were resistant at least to one of the SLDs tested. The results showed that 37.5% of the 40 MDR-TB isolates were resistant to ETH, 25% to CAP, 5% to AMI, 5% to KM, 5% to OFX, and 2.5% to CFZ. Mono-resistance to ETH, CAP, OFX or KM was detected in 10 (25%), 4 (10%) 1

(2.5%) and 1 (2.5%) strains, respectively. Mono-resistance to CFZ or AMI was not detected. Resistance to two drugs in different combinations was found in three (7.5%) strains, resistance to three drugs was found in two (5.0%) strains, and resistance to four drugs was found in only one (2.5%) strain. One (2.5%) of MDR-TB isolate cases was defined as extensively drug-resistant tuberculosis (XDR-TB). Simultaneous resistance to all SLDs tested was not observed. *M. tuberculosis* H37Rv strain used for internal quality control was susceptible to all SLDs tested.

Table. Resistance of the 40 MDR *M. tuberculosis* strains to second-line anti-tuberculosis drugs.

	No of strains	%
SUSCEPTIBLE TO ALL DRUGS	18	45.0
ANY RESISTANCE	22	55.0
- ethionamide (ETH)	15	37.5
- capreomycin (CAP)	10	25.0
- amikacin (AMI)	2	5.0
- kanamycin (KM)	2	5.0
- ofloxacin (OFX)	2	5.0
- clofazimine (CFZ)	1	2.5
MONO-RESISTANCE	16	40.0
- ethionamide (ETH)	10	25.0
- capreomycin (CAP)	4	10.0
- amikacin (AMI)	0	0.0
- kanamycin (KM)	1	2.5
- ofloxacin (OFX)	1	2.5
- clofazimine (CFZ)	0	0.0
OTHER PATTERNS	6	15.0
- CAP + ETH	2	5.0
- CAP + CFZ	1	2.5
- CAP + ETH + OFX*	1	2.5
- CAP + ETH + AMI	1	2.5
- CAP + ETH + AMI + KM	1	2.5
NUMBER OF DRUGS RESISTANT TO:		
2 drugs	3	7.5
3 drugs	2	5.0
4 drugs	1	2.5
5 drugs	0	0.0
all drugs	0	0.0

* This is a pattern of extensively drug-resistant tuberculosis (XDR-TB).

Discussion

Despite recent progress in global control efforts, TB remains a major public health burden in most developing countries, and the total number of new TB cases is still rising slowly. There were an estimated 8.8 million new TB cases in 2005, with 7.4 million in Asia and sub-Saharan Africa (10). Instead of being eradicated, drug-resistant strains have evolved and have been documented in every country surveyed. Once a strain of *M. tuberculosis* develops resistance to isoniazid and rifampin, it is defined as MDR-TB. According to the World Health Organization (WHO) reports, MDR-TB is a slowly increasing health problem (1). The incidence of MDR-TB in the Aegean region of Turkey was found to be 6.8% (11), while the worldwide mean of the incidence of MDR-TB is 1.7% (1).

The treatment of MDR-TB becomes difficult since SLDs must be used, which are less potent and not as well tolerated as first-line agents. While this is often done without testing the susceptibility of MDR strains to SLDs, susceptibility testing is important in order to determine an effective treatment regimen and to avoid further development of resistance in patients with MDR-TB (5,6,12). Second-line treatment can be individualized, based on *in-vitro* drug resistance, or standardized. Even with standardized protocols, there is a need for accurate resistance data for SLDs after the preliminary drug resistance surveys are completed. Although individualized treatment is probably best, the need for reliable but costly laboratory facilities for DST means that standardized SLD treatment has been advocated for use in middle- and low-income countries. The latter strategy requires detailed knowledge of likely drug-resistance patterns, particularly for MDR-TB strains, which requires recent regional surveys or surveillance of resistance (13).

There are few articles examining the resistance to SLDs in Turkey. They show that the rate of susceptibility to SLDs varies in different parts of the country. However, there are differences in methods employed for DST, including the critical concentrations of drugs and critical proportions of resistance used in these studies (14-17). The quality of susceptibility testing results is clearly an important issue. DST for both primary and secondary anti-TB drugs with the broth-based radiometric BACTEC 460 TB system is well established and is considered the "gold standard" (4,18). In the present study, BACTEC 460 TB system and the drug concentrations

recommended by Pfyffer et al. (3) and accepted internationally (19) were used to perform susceptibility testing with the SLDs.

In the present study, we analyzed resistance to AMI, CAP, KM, OFX, ETH, and CFZ of MDR *M. tuberculosis* strains isolated from patients with TB in the Aegean region of Turkey. At 37.5%, ETH resistance was the highest among all SLDs tested. This result is not surprising, since cross-resistance between ETH, a derivative of isonicotinic acid, and isoniazid can occur (20). This may explain why the rate of resistance to ETH is high in this study. However, the fact that all tested isolates were resistant to isoniazid but only 37.5% were resistant to ETH might suggest that the mutations leading to drug resistance are located in different regions of the genome (21,22).

The rate of resistance to ETH in MDR-TB cases was also found to be high by different studies from Turkey (56.2%, 38.6%, 22.0%, 32.8%) (14-17) and from other regions of the world (48.6% in Argentina, 21.2% in Thailand) (21,23), while all MDR-TB cases in Ethiopia (24) were sensitive to ETH. Toungoussova et al. (6) found very high rate of resistance to ETH in MDR-TB in Russia (92.2%) and suggested that ETH was not readily available during the economic crisis in Russia and this might have created the necessary conditions for the emergence of resistance to ETH.

We tested OFX as a representative of the fluoroquinolones. Of 40 MDR-TB strains, only 2 (5.0%) were resistant to OFX. One of strains resistant to the OFX was also resistant to ETH and CAP. Similarly, in most previous studies from Turkey and other countries, OFX was found to be one of the most active drugs against MDR-TB (1.3% in Russia, 9.1% in Thailand, 4.0% in Turkey) (6,23,16).

AMI and KM are aminoglycoside antibiotics that bind to 16S rRNA as their target in a small ribosomal subunit and subsequently inhibit protein synthesis (23). We selected both AMI and KM for testing. Resistance to AMI or KM was found to be 5.0%. One of two strains resistant to KM was also resistant to AMI, ETH and CAP, while the other strain was resistant only to KM. Both strains resistant to AMI were also resistant to ETH and CAP. *M. tuberculosis* can develop resistance to KM as a result of cross-resistance between the aminoglycosides, but the binding site of each of the aminoglycosides may

be different; therefore, the ribosomal mutations that mediate resistance to aminoglycosides are likely to be drug-specific (25).

CAP, the basic peptide antimicrobial agent with a mechanism of action similar to that of the aminoglycosides, is sometimes used in combination therapy for the treatment of MDR-TB (19,20). In the present study, the proportion of strains resistant to CAP (25%) was higher than resistance to the other aminoglycosides, KM and AMI. Only two of 10 strains resistant to CAP were also resistant to AMI. Di Perri et al. (25) reported that CAP-resistant strains are not usually resistant to AMI.

In most of the previous studies, a high rate of susceptibility to aminoglycosides among MDR-TB cases has been reported (13-16,23). However, Toungousova et al. (6) from Russia and Morcillo et al. (21) from Argentina reported higher rates of resistance to KM (41.6% and 28.3%), which might be partially explained by the administration of these drugs or analogues to previously treated patients as monotherapy (6).

CFZ is an active drug against MDR-TB *in vitro* and in murine model, but no clinical data on its use for treating MDR-TB are currently available (12,19,25). In the present study, CFZ was active against the MDR strains of *M. tuberculosis* in nearly all cases (97.5%); only one isolate was resistant to CFZ and it was also resistant to

CAP. Similar rates for CFZ were reported from different parts of the world: 93.5% from Argentina (21), 100% from Ethiopia (24) and 97.1% from Russia (13).

Extensively drug-resistant tuberculosis (XDR-TB) is defined as resistance to at least rifampin and isoniazid from the first-line anti-TB drugs (the definition of MDR-TB) in addition to resistance to any fluoroquinolone, and to at least one of three injectable second-line anti-TB drugs used in TB treatment (CAP, KM, and AMI) (26). In the present study, one (2.5%) of the MDR-TB cases met the criteria for XDR-TB. This isolate was resistant to CAP, OFX and ETH.

In conclusion, according to the present study, aminoglycosides (KM and AMI) and OFX were rather active drugs against MDR-TB and they can be used in the treatment regimen in our region without any problems. ETH and CAP, however, were effective in 62.5% and 75.0% of the cases, respectively. Alternative drugs are necessary for patients that harbor strains resistant to ETH and CAP. In addition, XDR-TB does not yet seem to be a serious problem in the Aegean region of Turkey.

These results may be of use in our region to establish a standardized regimen for the treatment of patients with MDR-TB until reliable results of susceptibility testing for SLDs are available, after which individualized treatments with SLDs will be feasible.

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