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Drug resistance rates of *Mycobacterium tuberculosis* strains in Austria between 1995 and 1998 and molecular typing of multidrug-resistant isolates

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SUMMARY

In this study the drug resistance pattern of 3559 *Mycobacterium tuberculosis* strains isolated in Austria between 1995 and 98 was evaluated. Of these strains, 165 (4·6%) were resistant to one or more drugs, 113 (3·2%) to one of the tested drugs and 53 (1·5%) to two or more drugs. Monodrug resistance was observed most often to isoniazid (56 strains), followed by streptomycin (44 strains). Resistance to rifampicin or ethambutol alone was rarely seen (12 strains and 1 strain, respectively). Of the 53 strains resistant to 2 or more drugs, 25 were resistant to isoniazid and streptomycin, while 17 were multidrug resistant. Molecular typing revealed a large diversity among the multidrug-resistant strains.

INTRODUCTION

An estimated 8 million new cases of tuberculosis are reported to occur per year, making it one of the most frequently encountered infectious diseases in the world [1]. In western European countries the frequency of tuberculosis has been steadily declining over recent decades. In nearly all of these countries the tuberculosis notification rate is now less than 20 cases per 100000 inhabitants [2]. However, the incidence of tuberculosis in industrialized countries is strongly influenced by the migration of people from high-prevalence countries [3] and also reflects the results of national tuberculosis programmes. The treatment of this disease is mainly based on five drugs. Therefore, drug-resistant tuberculosis is a significant threat to tuberculosis control programmes. Since the beginning of the 90s an increase in *Mycobacterium tuberculosis* strains resistant to one or more of these antibiotics has been reported from several countries in the world [4–7]. Especially multidrug-resistant *M. tuberculosis* (MDR MTB) strains, defined as those being resistant to at least isoniazid and rifampicin (8), the two most potent drugs in anti-tuberculosis treatment, were demonstrated to be a threat to public health (9).

Preventive strategies to cope with the problem of MDR MTB strains are designed to hinder the spread of these strains, which requires control measures and also treatment guidelines such as directly observed therapy. The latter has been shown to have a positive influence on the rate of drug-resistant strains [10, 11].

With the start of the 'Euro TB' project in 1995 [12], an individual notification system with a compulsory reporting system either from the clinician or from the

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laboratory was launched in Austria. Laboratories were asked to report full susceptibility testing and, in case no susceptibility testing of the first isolate was performed, strains were ordered to be sent to the National Reference Centre for Mycobacteria for susceptibility testing. This ensured that, in the period 1995–8, more than 99% of all bacillary tuberculosis strains were subjected to resistance testing.

The aim of this study was to analyse the rate of resistance of all *M. tuberculosis* strains isolated in 1995–8 taking into account nationality and age of the patients and, furthermore, to determine the aetiology of MDR MTB strains in Austria. Molecular typing of all multidrug-resistant strains was performed in order to see whether these strains are spreading.

MATERIALS AND METHODS

Strains

In each *M. tuberculosis* strain isolated for the first time, resistance testing was performed. For all strains which were resistant to one or more drugs, confirmation testing was performed at the National Reference Centre for Mycobacteria.

The three first-line anti-tuberculosis drugs, namely isoniazid (INH), rifampicin (RMP) and ethambutol (EMB), were tested in 100% of the strains; streptomycin (SM), which is not commonly used in Austria, in 91%; and pyrazinamide (PZA), which was only tested from 4 laboratories, in 69% of all tested strains. Resistance testing was performed in accordance with the proportion and/or Bactec 460 radiometric method [13, 14]. For Bactec susceptibility testing the final concentrations of INH, RMP, EMB, SM and PZA were 0·2, 2·0, 3·75, 3·0 and 100 μg/ml, respectively.

Collection of data

All laboratories performing drug resistance testing (nine such laboratories exist in Austria) sent in records of name, surname, date of birth, address and results of resistance testing on a quarterly basis to the National Reference Centre for Mycobacteria. All these records were entered into a database based on Epi Info, version 6 (Centres for Disease Control and Prevention, Atlanta, USA).

Besides the laboratory database, an individual notification system for all patients suffering from tuberculosis also exists in Austria. In the latter database the individual's name, surname, date of birth, citizenship, site of infection, beginning and end of treatment, and former treatment of tuberculosis are recorded.

Spoligotyping of strains

The spoligotyping method is based on *in vitro* amplification of the DNA of the highly polymorphic Direct Repeat (DR) locus in the *M. tuberculosis* chromosome (15). This region has a characteristic organization with conserved 36 bp DR sequences interspersed with variable spacers. The PCR-amplified spacer DNA is hybridized to immobilized oligonucleotides representing each of the spacer DNA sequences.

DNA was extracted from the strains cultured on Loewenstein–Jensen slants according to the DNA preparation procedure of the Amplicor *M. tuberculosis* PCR kit, as described in detail earlier [16]. For PCR a Robocycler with hot top assembly (Stratagene Europe, Amsterdam, The Netherlands) was used. The amplification reaction was performed in a final volume of 50 μl containing 1 μl template DNA (20 ng), 20 pmol of each primer, 2·5 U of AmpliTaq Gold (Perkin–Elmer, Foster City, CA, USA), 0·2 mM of each dNTP (Perkin–Elmer) and 5 μl GeneAmp 10 × buffer (Perkin–Elmer). The primers DRa (5′-GGT TTT GGG TCT GAC GAC) and DRb (5′-CCG AGA GGG GAC GGA AAC) were synthetized by Codon Genetic Systems (Vienna, Austria). Primer DRa was biotinylated at the 5′ end. Cycling conditions were 10 min at 95 °C, then 1 min at 95 °C, 1 min at 55 °C, 30 sec at 72 °C for 30 cycles and finally 10 min at 72 °C. The amplified DNA was hybridized with 43 oligonucleotides, corresponding to the spacer sequences of *M. tuberculosis* H37Rv and *M. bovis* BCG P3, being immobilized on a membrane delivered by Isogen Bioscience (Maarssen, The Netherlands). Hybridization was performed according to the instructions of the manufacturer using a 45-lane blotter (Miniblotter MN45; Immunetics, Cambridge, MA, USA). Detection of bound DNA fragments was performed by incubating with streptavidin-POD conjugate (Boehringer–Mannheim, Mannheim, Germany) and ECL detection reagents (Amersham, Bucks, UK), followed by exposure to ECL hyperfilm (Amersham) for 20 min. DNA typing using RFLP analysis [17] was performed according to the method previously described in detail [18].

RESULTS

In the period 1995–8, 3559 *M. tuberculosis* strains from newly registered patients could be cultured. A total of 165 (4·6%) of these strains were resistant to one or more drugs (Table 1), 113 (3·2%) were resistant to one of the tested drugs and 53 (1·5%), to two or more drugs. Resistance to a single drug was observed most often with INH (56 strains or 1·6% of all strains resistant to single drugs), followed by SM (44 strains or 1·2%). Drug resistance to RMP alone was only seen in 12 strains and to EMB in only 1 strain. Analysing resistance to single drugs according to citizenship and a previous history of tuberculosis, INH resistance was found in 7 out of 41 patients with Austrian citizenship and a former history of tuberculosis, but in no patient out of 15 with foreign citizenship. Of the 44 SM-resistant strains, 30 were cultured from Austrian patients. One patient was known to have received former anti-tuberculosis treatment. Of the 14 patients with foreign citizenship, none had received former treatment. Acquired drug resistance to RMP alone could be assumed in 4 of 6 patients with Austrian citizenship and in 1 of 6 with foreign citizenship.

The age of Austrian patients from whom strains resistant to single drugs could be cultured was higher than the age of such patients with foreign citizenship. The mean age of Austrians with strain resistant in INH , SM or RMP alone was 57, 55 and 56 years, respectively, while that of patients with foreign citizenship was 29, 29·5 and 32 years, respectively. Of the 53 isolates resistant to 2 or more drugs, 2 were resistant to all 5 tested antibiotics, 1 to 4, and 7 to 3 antibiotics. Of the 44 strains resistant to 2 drugs, resistance to INH and SM was encountered most often (Table 2).

MDR MTB strains in accordance with the WHO definition [8] were found in 17 patients. Of the 17 MDR MTB strains, 11 were resistant only to INH and RMP, 3 to INH, RMP and EMB, 1 to INH, RMP, EMB and SM, and 2 to all 5 tested drugs (Table 3). Nine of these 17 patients were from Austria and 8 from foreign countries. Of the 9 Austrians, 8 had formerly received anti-tuberculosis drug treatment for longer than 1 month. In 6 patients a positive tuberculosis culture from earlier years was found. Four of the strains showed a sensitive susceptibility pattern at that time, 1 was resistant only to INH and 1 was resistant to all 5 drugs tested. In the latter case, the first resistance testing was done 15 years after the first documented diagnosis of tuberculosis. Of the patients with foreign citizenship, former tuberculosis was only demonstrated in one. At the time the disease had been diagnosed in the past, the patient had a fully sensitive strain. In all other patients, the former history was not known, since they had been staying in Austria for a relatively short period (mean, 2·7 years). None of the patients with MDR tuberculosis had proven HIV infection. However, as HIV testing is not mandatory in Austria, a conclusive statement in this regard cannot be made.

Spoligotyping of all 17 MDR MTB isolates revealed only 2 with an identical pattern (Fig 1, lanes 7 and 18). However, molecular typing of those two isolates by RLFP analysis showed distinct pattern (data not shown).

Table 1. *Resistance to one drug in 3559* M. tuberculosis *strains*

Anti-tuberculosis drugs*	Number of strains resistant (%)
INH	56/(1·6)
RMP	12/(0·3)
PZA	0
EMP	1/(0·03)
SM	44/(1·2)

* INH, isoniazid; RMP, rifampicin; PZA, pyrazinamide; EMB, ethambutol; SM, streptomycin.

Table 2. *Resistance to two or more drugs in 3559* M. tuberculosis *strains* (*except for MDR MTB*)

Anti-tuberculosis drugs*	Number of strains resistant (%)
INH + EMB + SM	3/(0·08)
INH + SM	25/(0·7)
INH + EMB	1/(0·03)
RMP + SM	4/(0·1)
RMP + EMB	1/(0·03)
EMB + SM	2/(0·06)

* INH, isoniazid; RMP, rifampicin; PZA, pyrazinamide; EMB, ethambutol; SM, streptomycin.

DISCUSSION

In a survey of drug resistance of *M. tuberculosis* strains performed in Austria in 1992 and 1993, which was limited to the eastern part of the country (19), the overall rate of resistance was 6·9% and, for MDR

Table 3. *Seventeen patients with multidrug-resistant* M. tuberculosis *strains isolated from 1995–8 in Austria*

Male/ female	Age	Resistance pattern*	Citizinship/year of entering Austria	History of tuberculosis/year of first treatment	Previous isolation of *M. tuberculosis* Year/resistance
Male	41	INH, RMP	Austria	Yes/1992	1992
Female	45	INH, RMP	Austria	Yes/1994	1994/INH
Female	55	INH, RMP	Austria	Possible	No
Male	35	INH, RMP	Pakistan/1993	No	No
Male	43	INH, RMP, PZA, EMB, SM	Austria	Yes/1960	1975/INH, RMP, EMB
Male	33	INH, RMP	Turkey/1989	Yes/1992	1992/none
Male	4	INH, RMP, EMB	Austria	No	No
Male	32	INH, RMP	Turkey/1990	No	No
Male	45	INH, RMP	Austria	Yes/1987	1987/none
Female	50	INH, RMP, EMB, SM	Austria	Yes/1991	No
Male	26	INH, RMP	Africa/1996	No	No
Male	57	INH, RMP	Austria	Yes/1975	1975/none
Male	35	INH, RMP, EMB	Bosnia/1996	No	No
Male	32	INH, RMP, PZA, EMB, SM	Russia/1998	No	No
Female	32	INH, RMP, EMB	Austria	Yes/1984	No
Female	23	INH, RMP, EMB	Turkey/1997	No	No
Female	27	INH, RMP	India/1992	No	No

* INH, isoniazid; RMP, rifampicin; PZA, pyrazinamide; EMB, ethambutol; SM, streptomycin.

MTB, it was 1·5%. Compared to these data there was a slight decrease in the rate of drug resistant strains in Austria. This may be due to the fact that the survey in 1992–3 covered only one part of Austria. According to the age-dependent incidence curve (data not shown), strains resistant to single drugs were present in a higher age group in patients with Austrian citizenship than in those with foreign citizenship. Among patients with Austrian citizenship there was no difference regarding the age group in which resistance to various single drugs was found. For several years SM has not been used as a first-line anti-tuberculosis drug, therefore, SM resistance was expected to be present in a higher age group, as a sign of reactivation of an old tuberculosis. This, however, could not be demonstrated.

The frequency of MDR MTB strains, which could be demonstrated in 0·5% of all tested strains, was comparable with that in other industrialized countries [8, 20]. Analysing the clinical reports of these patients it could be shown that, with the exception of one, all of the nine Austrian patients previously had undergone anti-tuberculosis treatment for longer than 1 month indicating that the majority had acquired MDR tuberculosis. The main reason for the interruption of treatment was lack of compliance. An initial MDR tuberculosis was found only in one patient, a boy aged 4 years in whom the source of infection could not be detected.

Analysing the records of the patients with foreign citizenship, only one case of former tuberculosis with a sensitive strain was documented in 1992. All other patients revealed no history of tuberculosis. The mean duration of the patients' stay in Austria was 2·7 years. Since this was a rather short period, it may be assumed that the infection had been acquired abroad. All these patients were originally from countries with a high rate of *M. tuberculosis* resistant strains.

The high diversity of MDR MTB strains, as revealed by spoligotyping and RLFP analysis, clearly indicates that MDR MTB strains, which have not been imported to Austria, are due to poor compliance. A recent study from Scotland – a country with a low incidence of MDR MTB strains – reported similar findings [21].

In conclusion, the resistance rate of *M. tuberculosis*, including MDR MTB strains, is low in Austria. With respect to cases infected with MDR MTB strains in the period 1995–8, no cross-infection was registered. Therefore, at the present time, resistance to single drugs and multidrug-resistance represent no significant threat to public tuberculosis programmes. However, monitoring of drug susceptibility patterns together with national guidelines for therapy such as

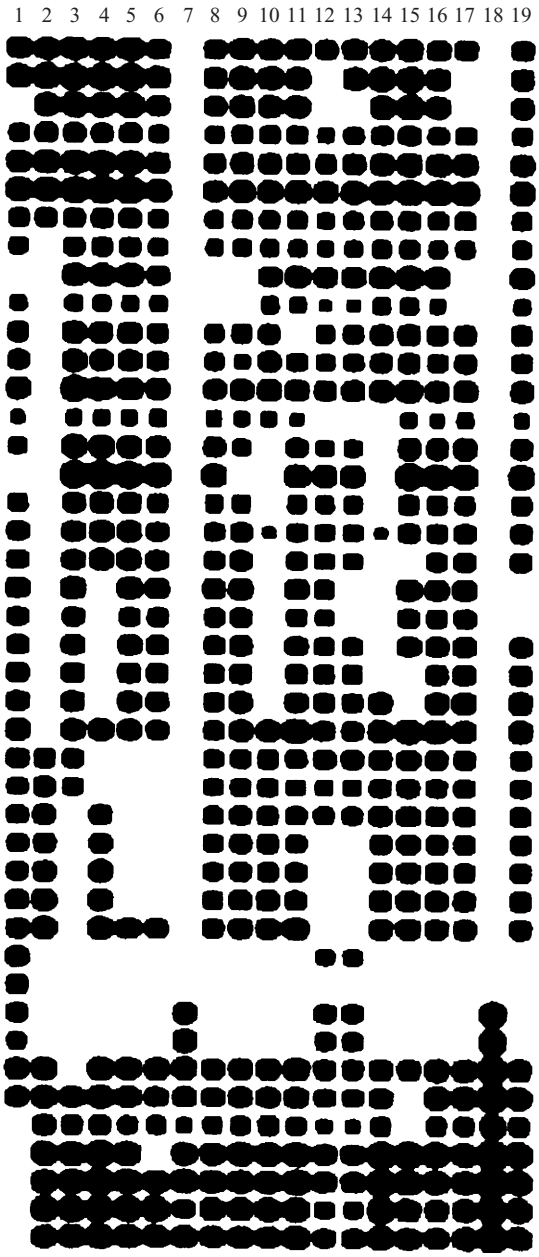

Fig. 1. Spoligotyping of MDR MTB strains isolated between 1995 and 1998 in Austria: Lanes 1 and 19 represent the spoligotypes of the references strains *M. bovis* BCG and *M. tuberculosis* H 37Rv, respectively; Lanes 2–18 represent the spoligotypes of 17 MDR MTB isolates.

directly observed therapy are important measures to optimize tuberculosis control and thus reduce the development of drug resistance.

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