INT J TUBERC LUNG DIS 8(12):1448–1457

Molecular epidemiology of drug-resistant *Mycobacterium tuberculosis* strains isolated from patients with pulmonary tuberculosis in Poland: a 1-year study

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SUMMARY

OBJECTIVE: To characterise drug-resistant *Mycobacterium tuberculosis* strains isolated in Poland and to estimate the amount of recent transmission in the population.

DESIGN: *M. tuberculosis* strains isolated from 251 patients with resistant pulmonary tuberculosis in Poland in 2000 were analysed by spoligotyping and IS*6110* DNA fingerprinting. Part of the strains was also characterised by sequencing of the *rpoB*, *katG* and/or the regulatory region of the *inhA* gene.

RESULTS: Using combined spoligotyping/IS*6110*-RFLP defined clusters, 29% of the strains were clustered, suggesting possible recent transmission. In some cases, transmission links among strains in clusters could be confirmed by epidemiological data and in addition, for most of the strains, by analysis of the mutations associated with resistance to rifampicin and/or isoniazid. Younger age, sex, immigration and history of previous treatment were not associated with clustering, whereas multidrug-resistant disease was more likely to cluster. Strains of the Beijing family could also be found in Poland, although with a much lower frequency than in the neighbouring countries.

CONCLUSION: Transmission of drug-resistant *M. tuberculosis* strains was demonstrated, which might contribute to the emergence of drug-resistant tuberculosis in Poland.

KEY WORDS: RFLP typing; spoligotyping; drug resistance; *M. tuberculosis*; Poland

EACH YEAR, nearly 3.5 million new cases of infectious tuberculosis (TB) occur worldwide.[1] The problem is becoming more critical with the emergence and spread of drug-resistant and multidrug-resistant (MDR, resistance to at least isoniazid [INH] and rifampicin [RMP]) strains of *Mycobacterium tuberculosis*. Poland has a relatively low incidence of TB compared with other eastern European countries, with 27.6 cases per 100 000 population in 2001. However, the prevalence of primary drug resistance increased twofold from 3.6% in 1997 to 6.1% in 2000, and a twofold increase in primary multidrug resistance was observed during this 3-year period (0.6% vs. 1.2%).[2]

M. tuberculosis strains from various geographical origins have been characterised by using restriction fragment length polymorphism (RFLP) analysis with the organism's repetitive DNA element IS*6110*. International consensus has been reached to use this method for subtyping *M. tuberculosis* isolates to facilitate comparison of patterns among different laboratories worldwide.[3] RFLP typing has proven useful for detection of outbreaks,[4] control of laboratory cross-contamination,[5] and population-based epidemiological studies of TB.[6,7]

Alternative polymerase chain reaction (PCR) based approaches have also been developed. One such procedure is spoligotyping (spacer oligonucleotide typing), based on DNA polymorphism of the *M. tuberculosis* direct repeat (DR) chromosomal region.[8] The method is rapid, easy to perform, and is now often used in addition to IS*6110* RFLP analysis. Spoligotyping has been applied to differentiate strains with highly similar IS*6110* RFLP patterns or strains with fewer than five IS*6110* copies.[9]

M. tuberculosis strains with the same IS*6110* RFLP pattern may exhibit different resistance profiles to anti-tuberculosis drugs. Resistance to RMP is conferred by specific point mutations and small insertions or deletions in an 81-bp region of the *rpoB* gene encoding the β subunit of the RNA polymerase.[10–12]

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Article submitted 24 November 2003. Final version accepted 19 April 2004.

By contrast, resistance to INH is associated with a variety of mutations in several genes, such as that coding for catalase-peroxidase (*katG*)[13] and the regulatory region of enoyl-ACP reductase (*inhA*),[14] which are affected most frequently. Knowledge of resistance mutations can further be used for a detailed analysis of clustered *M. tuberculosis* isolates providing additional information on the strains' relationship, as was shown for RMP-resistant (RMPr) isolates.[11,12]

The aim of the present study was to extend previous results[15] and to characterise drug-resistant *M. tuberculosis* strains isolated in Poland. *M. tuberculosis* strains isolated in 2000 from 251 patients with drug-resistant pulmonary TB (PTB) were analysed by IS*6110* RFLP and spoligotyping. The strains were also partly characterised by sequencing of the *rpoB*, *katG* and/or the regulatory region of the *inhA* gene.

MATERIALS AND METHODS

Patient characteristics

The *M. tuberculosis* strains examined in this cross-sectional study were isolated from 251 patients with PTB in Poland in 2000 during the second national survey of drug resistance. In this survey, 186 primary resistant cases and 111 resistant cases among previously treated individuals were identified.[2] Of these, 164 (88.2%) primary resistant cases and 87 (78.4%) previously treated patients who excreted drug-resistant bacilli were enrolled in the study.

Data on the patients' past medical history of TB, chemotherapeutic regimens, human immunodeficiency virus (HIV) status, and standard demographic data were registered. Patients originated from different regions of the country; most were of Polish origin (96.4%). Nine foreign-born patients (3.6%) were from Romania ($n = 2$), Russia ($n = 2$), Ukraine ($n = 1$), Armenia ($n = 1$), Pakistan ($n = 1$), Vietnam ($n = 1$), and South Korea ($n = 1$). The patients were aged between 16 and 91 years and 78% were aged between 20 and 60 years. Most of the patients (73.3%) were men. None were HIV-positive.

Bacterial strains

The 251 drug-resistant *M. tuberculosis* strains analysed in the present study were provided by the National Tuberculosis Reference Laboratory in Warsaw. Primary isolation, identification and drug susceptibility testing were performed using the Löwenstein-Jensen (LJ) medium and the BACTEC 460-TB system (Becton-Dickinson, Sparks, MD, USA), as reported earlier.[2]

RFLP analyses

All strains were investigated by IS*6110* RFLP analysis using the international standard technique.[3] The IS*6110* RFLP patterns were inspected visually, scanned and analysed using GelCompar II computer software, version 2.5 (Applied Maths, Sint-Martens-Latem, Belgium). The IS*6110* patterns were compared using the Dice coefficient with the parameter settings at 1.0% band position tolerance with optimisation. A molecular cluster was defined as a series of isolates exhibiting 100% identical IS*6110* banding patterns.

Spoligotyping

Purified chromosomal DNA from RFLP typing was available for the analysis. Spoligotyping was performed using a commercially available kit (Isogen Bioscience BV, Maarssen, The Netherlands) according to the manufacturer's instructions as previously described.[8]

Database comparison

The international spoligotyping database project (SpolDB) is an open project aimed at describing the worldwide genetic diversity of tuberculosis genomes. It currently involves around 80 co-investigators worldwide. A third update (SpolDB3), describing 817 alleles representative of 11 308 patients, was released in 2003.* A fourth release, describing more than 1500 alleles representative of 28 000 patients worldwide, is in preparation. By introduction of the Polish spoligotype file ($n = 251$), a query was made against an updated SpolDB3 database containing a total of 26 676 isolates, split into 1547 shared types (STs) totalling 24 214 isolates plus 2462 orphan alleles. After introduction of the file, the updated SpolDB3 contained 26 927 isolates split into 1560 shared types (13 new clusters were created), totalling 24 446 isolates and 2481 orphan alleles.

PCR amplification

DNA extraction for PCR was performed by scraping a loopful of the organisms from a LJ slant. The bacteria were suspended in 1 ml of sterile water and lysed by boiling for 20 min. The cells were centrifuged (12 000 × g for 5 min), then frozen overnight at −20°C. The supernatant was used as a template for amplification.

Nucleotide sequences of the primers used for amplification of the *rpoB* and *katG* genes were described by Telenti et al.;[16] primers for amplification of the *inhA* regulatory region were described by Gonzalez et al.[17] Amplification reactions were performed with 50 μl volumes containing 2.5 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 1 μM of each primer, 1 U of HotStart *Taq* DNA polymerase (Qiagen GmbH, Hilden, Germany), and 7 μl of template DNA. In addition, the reaction mixture for the *rpoB* amplification included 8 μl of Q solution (Qiagen). The cycling parameters were 95°C for 15 min to activate the HotStart *Taq* DNA polymerase, followed by 35 amplification cycles at 94°C for 30 s; 62, 64 or

* www.pasteur-guadeloupe.fr/tb/spoldb3

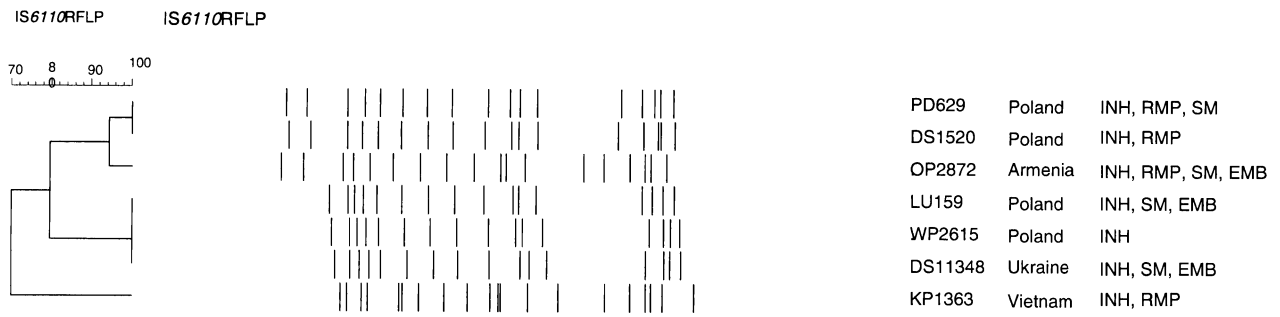

Figure 1 IS*6110* RFLP patterns of the seven *M. tuberculosis* strains of the Beijing genotype and the corresponding dendrogram. The numbers of strains, drug resistance profiles and origin of patients are indicated on the right. The similarity between the patterns is indicated as a percentage above the dendrogram. INH = isoniazid; RMP = rifampicin; SM = streptomycin; EMB = ethambutol; RFLP = restriction fragment length polymorphism.

67°C (annealing temperatures for *rpoB*, *inhA* and *katG*, respectively) for 30 s, and 72°C for 45 s in an MJ Research thermocycler (MJ Research Inc, Waltham, MA, USA). PCR products were examined by gel electrophoresis, purified using QIAquick PCR Purification kit (Qiagen) and applied in sequencing analysis.

DNA sequencing

The PCR primers were also used for direct sequencing of both strands of the amplification products. Sequencing reactions were carried out with an ABI Prism 377 automated DNA sequencer and corresponding kits from the same manufacturer (Applied Biosystems, Foster City, CA, USA). The sequences obtained were compared to their respective wild-type sequences using the Blast2 Sequences computer programme.*

Statistical analysis

The characteristics of patients with clustered strains and those with nonclustered strains were compared. With categorical variables the χ^2 test was applied, or, when expected values were less than 10, the V-square test was performed. Differences were considered as significant if $P < 0.05$.

RESULTS

RFLP analysis of drug-resistant M. tuberculosis *strains*

The 251 strains were analysed by RFLP with IS*6110* as a hybridisation probe. The IS*6110* fingerprint patterns generated were highly variable. The number of IS*6110* copies per strain varied from 5 to 20. The majority, 203 (81%) of the strains, contained 6–11 copies, with a mean of 10 bands. Of 203 IS*6110* fingerprint patterns observed, 179 were unique, indicating epidemiological independence. However, 72 (29%) strains clustered in 24 groups consisting of 2–8 isolates with identical IS*6110* RFLP patterns, presumably representing cases of recent transmission. Most of the clusters (67%) included pairs of isolates.

Four RFLP patterns, representing seven of the 251 strains (2.8%), shared the majority of the IS*6110* copies and clustered at a level of 70% similarity (Dice coefficient). In particular, two distinct patterns within this fingerprint group were shared, one by two strains and the other by three strains. By comparison with fingerprints described in the literature,[18] the RFLP patterns of this group were similar to the Beijing genotype. These strains harboured 16–20 copies of IS*6110*, while the remaining strains contained 5–17 copies (Figure 1).

Spoligotyping

The spoligotyping method was also used to confirm relationship of strains (Figure 2). A total of 91 different types were identified by spoligotyping in the 251 strains split into 192 clustered and 59 unique patterns. The types were compared with those contained in the updated version of the international spoligotyping database SpolDB3.[19] Thirty-two clusters comprising 192 (76.5%) clinical isolates and 59 unique profiles were detected; 25 (10%) of the 59 unique profiles were found to be truly unique and new, whereas the remaining 34 profiles had already been described elsewhere and were thus labelled according to previously identified ST numbers. Among the 32 clusters, four were specific for the setting studied: ST1555, ST1557, ST1561 and ST1594, made up of 2, 8, 2 and 2 clinical isolates, respectively. As these clusters are new and not found elsewhere, they are likely to represent either specific historical, recent or both transmission events in the setting studied. Ninety-six (38%) isolates were grouped into six clusters: ST53 (n = 34, 13.5%), ST50 (n = 19, 7.6%), ST47 (n = 16, 6.4%), ST1051 (n = 10, 4%, a cluster first described in Azerbaijan), ST1558 (n = 9, 3.6%, also found in Austria), and ST1557 (n = 8, 3.2%, not previously reported).

*Correlation between IS*6110 *RFLP patterns and drug resistance*

Of the 251 *M. tuberculosis* strains, 164 (65%) were isolated from new patients who had never been treated for TB, and 87 (35%) were obtained from

* www.ncbi.nlm.nih.gov/BLAST/

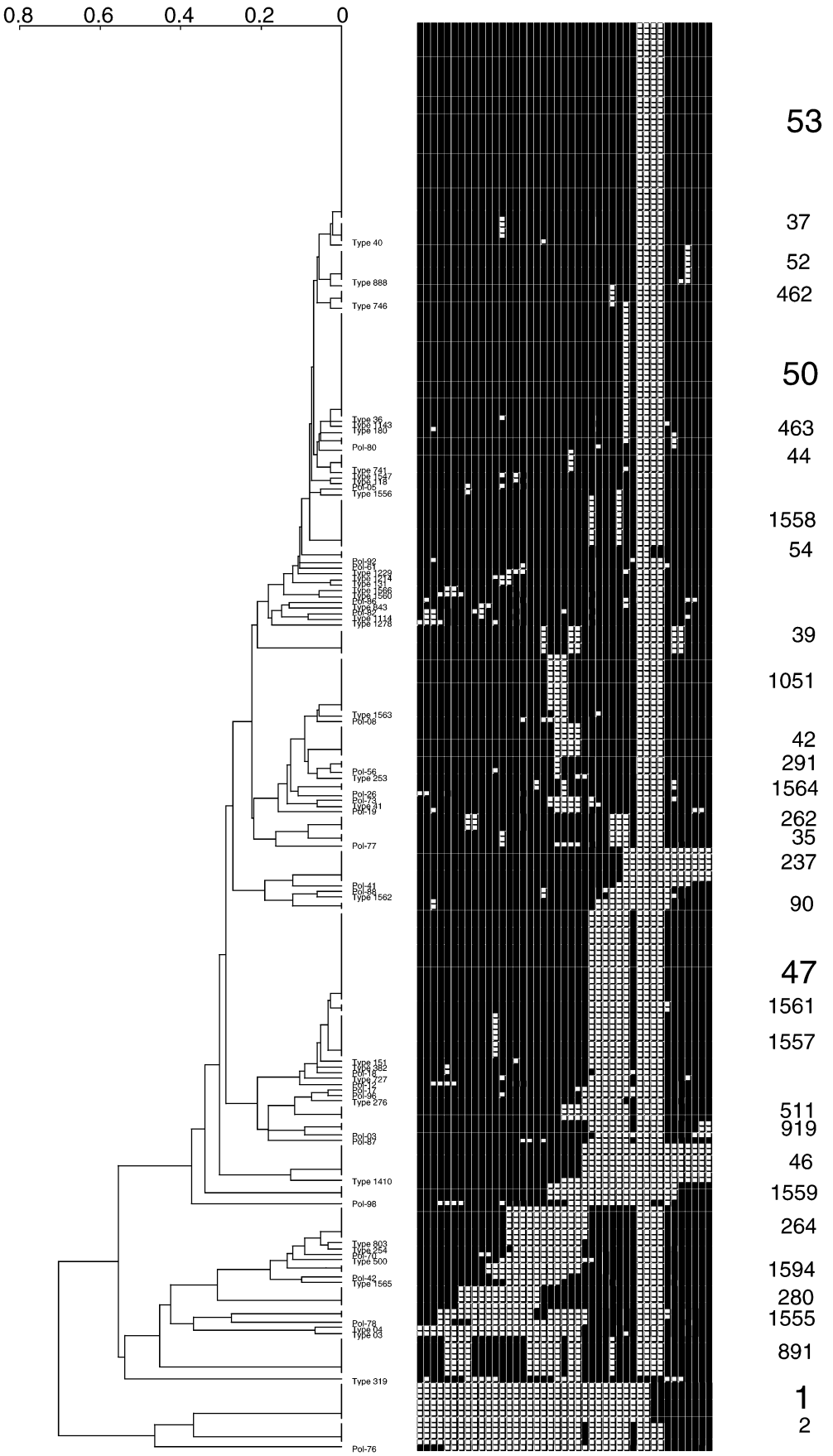

Figure 2 Dendrogram of Polish spoligotypes built using the Taxotron software, constructed following the user's manual (P A D Grimont, 2000, Institut Pasteur, Paris). The 1-Jaccard index was used for distance matrix building and the UPGMA (unweighted pair group method using arithmetic averages) for clustering. From left to right: dendrogram, index of unique isolates (either truly unique under the Pol-xx nomenclature or using the international shared type ST designation of these isolates), binary spoligotype matrix result, designation of the clusters (ST number). The 32 STs, totalling 192 isolates and 59 orphan isolates, are seen. Larger font is used to distinguish well known ubiquitous spoligotypes such as ST1 (Beijing), ST47 (Haarlem 1), ST50 (Haarlem 3), ST53 (T1 superfamily). ST = shared type.

Table 1 Drug resistance profiles of *M. tuberculosis* strains from Poland

Drug resistance profile	Resistant strains		Total *n* (%)
	Primary resistance *n* (%)	Acquired resistance *n* (%)	
Monoresistance			
INH	56 (34.1)	18 (20.7)	74 (29.5)
RMP	8 (4.9)	3 (3.4)	11 (4.4)
SM	51 (31.1)	12 (13.8)	63 (25.1)
Multidrug resistance			
INH, RMP	7 (4.3)	10 (11.5)	17 (6.8)
INH, RMP, EMB	1 (0.6)	5 (5.7)	6 (2.4)
INH, RMP, SM	7 (4.3)	18 (20.7)	25 (10.0)
INH, RMP, EMB, SM	14 (8.5)	9 (10.3)	23 (9.2)
Other profiles			
INH, SM	18 (11.0)	11 (12.6)	29 (11.6)
INH, EMB, SM	1 (0.6)	1 (1.1)	2 (0.4)
RMP, SM	1 (0.6)	0	1 (0.4)
Total	164	87	251

INH = isoniazid; RMP = rifampicin; SM = streptomycin; EMB = ethambutol.

previously treated patients (Table 1). Nearly one third of the strains were resistant to INH (29.5%) and to streptomycin (25.1%). Seventy-one strains (28.3%) were MDR, of which 29 (41%) comprised primary cases. Of the seven strains of the Beijing genotype, four (57%) showed MDR.

A total of 105 RMPr and/or INH-resistant (INHr) *M. tuberculosis* strains identified by conventional methods were further investigated by DNA sequencing to identify the mutations associated with resistance to these drugs. The analysed strains comprised 83 INHr and 64 RMPr strains (INH- and/or RMP-resistant, including 59 MDR strains). Amplicons containing the 81-bp polymorphic region of the *rpoB* gene (RMP resistance), and codon 315 of the *katG* gene and/or the regulatory region of the *inhA* gene (INH resistance) were analysed. The *rpoB* gene showed the highest polymorphism, with 19 different mutations. In the case of resistance to INH, 66% of the strains tested had a Ser315Thr mutation, while in the *inhA* regulatory region the only change was the nucleotide substitution −15C→T (14.5%).[20]

Among the 105 strains tested for polymorphism in the *rpoB*, *katG* and/or *inhA* loci, 58 showed unique IS*6110* RFLP patterns, confirming independent acquisition of resistance. The remaining 47 (45%) strains belonged to 15 clusters of two to eight strains (Table 2). Transmission of the same drug-resistant *M. tuberculosis* strain among the patients of one cluster or their infection by contact with one or more index patients could further be supported by the observation that in most clusters all of the strains exhibited the same polymorphism in RMPr, sometimes involving unique double mutations (e.g., cluster O in Table 2), and/or INHr-associated genes, and identical or very similar resistance profiles. Cluster O comprised seven epidemiologically-related patients, of whom five had primary and two had acquired MDR-TB. Polymorphism in drug resistance-related genes proved a very useful molecular tool in further refining links among those patients and the direction of transmission events. Only strains of the largest cluster, F, differed markedly in their drug resistance profiles. In these cases, TB infections may have been acquired several years before and the different drug resistance patterns of the strains may have developed during the patients' histories. Also, in only five clusters isolates had a different RMPr and/or INHr mutation, indicating independent acquirement of resistance to these drugs.

Characteristics of clustered patients and associated factors

Characteristics such as the patients' age and sex, birthplace, previous treatment for TB and resistance of strains to drugs were compared for the clustered (n = 72) and non-clustered (n = 179) patients (Table 3). The patients in clusters were more likely than the non-clustered patients to be infected with an MDR *M. tuberculosis* strain (50.0% vs. 19.6%, $P < 0.0001$). Resistance to a single drug was more common in patients not included in a cluster (67.0% vs. 38.9%, $P < 0.0001$). Other characteristics, as shown in Table 3, did not differ between the two groups.

Epidemiological investigations, based on patient contact tracing, have been performed for clustered patients (72 patients in 24 clusters). Definite transmission links (e.g., household or family contacts) could be verified for six patients in three clusters. For 29 members of 13 further clusters links were possible, but eight clusters (33%), including 37 patients (51.4%), remain unexplained.

DISCUSSION

We previously analysed the IS*6110* RFLP patterns of the relatively low number of 98 *M. tuberculosis* strains from TB patients in Poland from 1993 to 1995 in relation to their drug resistance profiles.[15] In the present report, we describe the molecular epidemiology of *M. tuberculosis* strains isolated from 85% of all patients with drug-resistant TB identified in 2000 during the second national drug resistance survey.[2] Primary drug resistance has been monitored in Poland since 1960 with decreasing frequency up to 1997. In 2000, a twofold increase in primary resistance rate (including MDR cases) was observed in comparison with 1997 (6.1 and 1.2%, respectively).[2] These findings show that drug-resistant TB may constitute an important problem in Poland in the near future.

Some molecular epidemiological studies recently demonstrated that the transmission of drug-resistant strains is no different from that of drug-sensitive strains, and recent transmission of drug-resistant strains was assumed to contribute substantially to the increase of TB.[21–23] We found that 29% of the patients with

Table 2 Characteristics of 15 IS*6110* RFLP-spoligotyping clusters among the 251 drug-resistant *M. tuberculosis* strains

Cluster/ST	Strain	Treatment history	Drug resistance profile	Polymorphism	
				rpoB	*katG* or *inhA*
A/53	1	+	INH, RMP	NM	Ser315Thr
	2	+	INH, RMP, SM	Asp516Val	Ser315Thr
B/1555	3	−	INH	ND	Ser315Thr
	4	+	INH	ND	SER315Thr
C/52	5	−	RMP	Ser531Trp	ND
	6	−	RMP	Ser531Trp	ND
D/42	7	−	INH, RMP	Ser531Leu	Ser315Thr
	8	+	INH, RMP, SM	Ser531Leu	ND
E/1558	9	−	INH, RMP, EMB	ND	Ser315Thr
	10	+	INH, RMP, EMB	Asp516Tyr	Ser315Thr
	11	−	INH, RMP, EMB, SM	Asp516Tyr	Ser315Thr
	12	+	INH, RMP, EMB, SM	Asp516Tyr	Ser315Thr
	13	+	INH, RMP, EMB	Asp516Tyr	Ser315Thr
	14	+	INH, RMP, EMB, SM	Asp516Tyr, Gln510His	Ser315Thr
	15	+	INH, RMP, EMB, SM	Asp516Tyr, Gln510His	Ser315Thr
F/1051	16	−	INH, RMP, EMB, SM	NM	Ser315Thr
	17	−	INH, RMP, SM	Ser531Leu	Ser315Thr
	18	−	INH, RMP	Ser531Leu	ND
	19	−	INH, RMP	Ser531Leu	ND
	20	+	INH, RMP, EMB, SM	Asp516Gly, Arg529Gln	ND
	21	+	INH, RMP, SM	ND	ND
	22	+	INH, RMP, SM	ND	ND
	23	−	SM	ND	ND
G/1*	24	+	INH, RMP	Ser531Leu	Ser315Thr
	25	+	INH, RMP, SM	Ser531Leu	Ser315Thr
H/1*	26	−	INH, EMB, SM	ND	Ser315THr
	27	+	INH, EMB, SM	ND	NM, Inh (C/T)
	28	−	INH	ND	ND
I/463	29	−	INH, SM	ND	NM
	30	+	INH, RMP, SM	Ser531Leu	NM, Inh (C/T)
J/1559	31	−	INH, SM	ND	NM
	32	−	INH, SM	ND	NM
K/264	33	−	INH	ND	Ser315Thr
	34	−	INH	ND	Ser315Thr
	35	−	INH	ND	Ser315Thr
	36	−	INH	ND	Ser315Thr
L/891	37	+	INH, RMP, SM	Ser531Leu	Ser315Thr
	38	+	INH, RMP, SM	Ser531Leu	Ser315Thr
	39	+	INH, RMP, SM	Ser531Leu	Ser315Thr
	40	−	INH, RMP, SM	Ser531Leu	Ser315Thr
	41	+	INH, RMP, SM	Ser531Leu	ND
M/1594	42	−	INH, RMP	Asp516Val	ND
	43	−	INH	ND	ND
N/2	44	−	INH, SM	ND	NM, Inh (C/T)
	45	+	INH, SM	ND	NM, Inh (C/T)
O/1557	46	−	NH, RMP, EMB, SM	Met515Ile, Asp516Tyr	Ser315Thr
	47	−	NH, RMP, EMB, SM	Met515Ile, Asp516Tyr	Ser315Thr
	48	−	NH, RMP, EMB, SM	Met515Ile, Asp516Tyr	Ser315Thr
	49	−	NH, RMP, EMB, SM	Met515Ile, Asp516Tyr	Ser315Thr
	50	−	NH, RMP, EMB, SM	Met515Ile, Asp516Tyr	Ser315Thr
	51	+	NH, RMP, EMB, SM	Met515Ile, Asp516Tyr	Ser315Thr
	52	+	NH, RMP, EMB, SM	Met515Ile, Asp516Tyr	Ser315Thr

* Clusters consisting of Beijing family strains.
RFLP = restriction fragment length polymorphism; ST = shared type in SpolDB3; + = treated; INH = isoniazid; RMP = rifampicin; NM = no mutation; SM = streptomycin; − = untreated; ND = not determined; EMB = ethambutol; Inh = mutation −15C→T in the *inhA* regulatory region (C/T).

drug-resistant PTB had *M. tuberculosis* isolates that belonged to clusters representing possible cases of recent transmission. The prevalence of clustering observed in this study was similar to that observed in Germany (33%),[24] and considerably lower than that reported in Estonia (49%)[22] and Archangel Oblast, Russia (59.7%).[23] It may be explained by lower prevalence of TB in Poland in comparison with these former Soviet Union (USSR) republics, where the prevalence of TB is extremely high. These studies also

Table 3 Characteristics of patients with PTB by clustered and non-clustered groups

Characteristic	Clustered (n = 72) n (%)	Non-clustered (n = 179) n (%)	Total (n = 251) n (%)	P
Age (mean 52 years)*				
⩽52 years	46 (65.7)	114 (66.3)	160 (66.1)	0.93 NS
>52 years	24 (34.3)	58 (33.7)	82 (33.9)	
Sex				
Male	55 (76.4)	129 (72.1)	184 (73.3)	0.48 NS
Female	17 (23.6)	50 (27.9)	67 (26.7)	
Birthplace				
Poland	70 (97.2)	172 (96.1)	242 (96.4)	0.66 NS
Other	2 (2.8)	7 (3.9)	9 (3.6)	
Previous treatment				
Yes	30 (41.7)	57 (31.8)	87 (34.7)	0.14 NS
No	42 (58.3)	122 (68.2)	164 (65.3)	
Drug resistance				
Single drug	28 (38.9)	120 (67.0)	148 (59.0)	<0.0001
Multidrug	36 (50.0)	35 (19.6)	71 (28.3)	

* Excluding 9 patients for whom data regarding age were not available.
PTB = pulmonary tuberculosis; NS = not statistically significant.

comprised a large proportion of genetically closely related Beijing genotype strains, associated with drug and multidrug resistance. In the present study, only 2.8% of the strains belonged to the Beijing genotype but they were more likely to be part of a cluster (71%).

The largest cluster in our study involved eight patients. In our previous report, the largest cluster included only three patients with MDR *M. tuberculosis* isolates.[15] The present finding of larger clusters of resistant strains indicates the existence of active transmission of these strains in Poland, contributing to the increased rates of resistance among new cases. Recent infection, as indicated by clustering, was not associated in our study with being male, younger age or having a history of previous anti-tuberculosis treatment. Similar results have been found in some previous studies,[7,25] but not in others.[6,26] Furthermore, no association was noted with immigration, simply due to the fact that in our study only nine cases were foreign-born. However, two (22%) of these were part of separate clusters, thus contributing to the transmission of resistant TB within the community. In previous studies where resistant strains were found in clusters, it was demonstrated that transmission of MDR strains had occurred between HIV-infected patients.[27] Our results are similar to other studies,[22,26] proving that transmission of MDR-TB is not limited to HIV-seropositive patients in an institutional setting, but occurs within a community.

Only 48.6% of the clustered cases had definite or possible epidemiological links, which is consistent with other reports in the literature.[6,7,28] A possible reason for this low concordance between molecular and conventional epidemiological findings might be that a portion of clustered patients were infected with strains prevalent in a given area and developed active PTB by chance during the study period. It has also been postulated that molecular clustering may not be due to recent transmission, as identical patterns could occur independently for isolates of different origins due to convergence of banding patterns.[29,30] Clustering rates should thus be interpreted with care. The clustering index according to Small et al.[31] among the strains analysed in this study was 19%, indicating that, if the proportion of molecular clustering of isolates truly reflects recent transmission of disease, almost one fifth of the active cases of resistant TB in Poland are due to recent transmission of the disease. The clustering index determined here is similar to that observed in some previous studies,[7,24] but is higher than in others.[32]

RFLP analysis has shown that *M. tuberculosis* strains with the same *rpoB*, *katG* and/or *inhA* alleles can have distinct IS*6110* RFLP patterns and that some strains with identical IS*6110* fingerprints can have different *rpoB*, *katG* and/or *inhA* alleles. Thus the same mutant, especially *rpoB*, genotypes can be produced by unrelated strains, and clonally related strains can have subclones bearing distinct mutant *rpoB* alleles. However, in most clusters all of the isolates had identical or very similar drug resistance profiles and the same mutations in the *rpoB* and *katG* genes and/or in the regulatory region of *inhA*. These results further confirm the close relationship of *M. tuberculosis* strains belonging to one cluster and transmission of the same drug-resistant strain among the patients of one cluster.

Spoligotyping, a PCR-based genotyping method developed in 1997,[8] was applied here for the first time as an additional molecular tool to differentiate *M. tuberculosis* isolates in Poland. In accordance with previous findings, spoligotyping was less discriminatory than IS*6110* RFLP.[8,9] A total of 91 spoligotypes

among 251 *M. tuberculosis* strains analysed were observed. However, 32 of these, totalling 47 (19%) clinical isolates, had spoligotypes that had not previously been submitted to the worldwide database. Four clusters (ST1555, ST1557, ST1561, ST1594), comprising 14 clinical isolates, had not been described elsewhere in the world among the 26 676 profiles from more than 120 countries submitted so far to the updated SpolDB3. The three most prevalent spoligotypes caused 27% of the resistant TB cases in Poland and are also among the most common in the world (ST53, 50 and 47).[19] Comparison of our spoligotype results with the updated international database provided a better picture of the global geographical specificity of the MDR clones found in Poland, and a detailed analysis will be published elsewhere. Globally, our results suggest that most of the clones found in Poland are historically and geographically linked to the rest of Europe by three geographical, demographical and historical components, which are (by decreasing importance): 1) links with central Europe through the Czech Republic and Austria, 2) links with the former USSR, and 3) links with northern Europe (Finland and Sweden). In particular, 10% of spoligotypes found in this study have been reported from Russia. These results confirm the importance of the current increased risk of TB disease spreading east to west through population flows from the former USSR countries to Western Europe through Poland, Austria and northern European countries, requiring an ongoing, robust, active TB control and surveillance system in this large European area.

Interestingly, strains of the Beijing family were demonstrated in Poland for the first time in the present study. The spread of strains of the Beijing genotype to Poland's eastern and western neighbours, Russia and Germany, was recently described.[23,24,33] In north-western Russia, 44.5% of the strains in the Archangel Oblast[23] and 60% of INHr strains around St Petersburg belonged to the Beijing genotype.[33] In Germany, strains of the Beijing family comprised 17% of resistant TB cases in 1995 and were mainly isolated from immigrants from former USSR countries.[24] Three of the seven Beijing genotype strains encountered in our study were isolated from patients originating from countries where the genotype is prevalent.

This study confirms and adds to previous results on molecular epidemiology of tuberculosis in Poland. Transmission (defined by the degree of clustering) of drug-resistant *M. tuberculosis* strains, including strains of the Beijing family, was demonstrated and seems to contribute to the emergence of drug-resistant TB in Poland. Molecular epidemiological analyses, including the use of polymorphism in RMPr and/or INHr-associated genes, proved a very useful adjunctive approach to classical epidemiological methods. They helped to identify transmission links and/or the direction of transmission events among patients who would have remained unrecognised by conventional contact investigation. The national TB programme in Poland is based on the DOTS strategy, resulting in a continuous decrease in TB infection and mortality rates. Active transmission of drug-resistant *M. tuberculosis* strains may have contributed to an unexpected increase in primary resistance, including MDR cases, observed in Poland in the 1997 and 2000 surveys. The increased rates of drug resistance may also be a function of immigration from areas of high resistance, such as Russia and other former USSR countries. Continued surveillance, including molecular epidemiology techniques, and appropriate therapeutic decisions should be undertaken to prevent the spread of drug-resistant and MDR-TB within Poland and, potentially, the other European Union countries.

Acknowledgements

The work performed in Poland was supported by a grant from the State Committee for Scientific Research (KBN, contract nos. PBZ030-15 and P05A14024). AS was supported by FEMS and NATO Science Fellowships at the Institute of Tropical Medicine, Antwerp, Belgium. This work was also supported by the Fund for Scientific Research of Flanders (Brussels, Belgium, grant no. G.0471.03 N).

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RÉSUMÉ

OBJECTIF : Caractériser les souches de *Mycobacterium tuberculosis* résistantes aux médicaments isolées en Pologne et estimer le niveau de transmission récente de la maladie dans la population.

SCHÉMA : Les souches de *M. tuberculosis* isolées chez 251 patients atteints de tuberculose pulmonaire à germes résistants en Pologne en 2000 ont été analysées par spoligotypage et par empreintes digitales de l'ADN par IS*6110*. Une partie des souches a été également caractérisée par séquençage des *rpoB*, *katG* et/ou de la région de régulation du gène *inhA*.

RÉSULTATS : L'utilisation combinée du spoligotypage et de la RFLP-IS*6110* a permis de définir des grappes dans 29% des souches, ce qui suggère la possibilité d'une transmission récente. Dans certains cas, les liens de transmission parmi les souches en grappes ont pu être confirmés par les données épidémiologiques et en outre, pour la plupart des souches, par l'analyse des mutations associées à la résistance à la rifampicine et/ou à l'isoniazide. On n'a pas noté d'association entre le développement de grappes et le jeune âge, le sexe, l'immigration ou les antécédents de traitement antérieur, alors que les maladies à germes multirésistants étaient plus susceptibles de se retrouver en grappes. Les souches de la famille Beijing ont pu être trouvées également en Pologne, quoiqu'à une fréquence beaucoup plus faible que dans les pays voisins.

CONCLUSION : On a pu démontrer la transmission de souches de *M. tuberculosis* résistantes aux médicaments, ce qui pourrait contribuer à l'émergence de tuberculoses à germes résistants en Pologne.

RESUMEN

OBJETIVO : Caracterizar las cepas de *Mycobacterium tuberculosis* farmacorresistentes aisladas en Polonia y calcular el nivel de transmisión reciente en la población.
MÉTODO : En 2000, se aislaron cepas de *M. tuberculosis* provenientes de 251 pacientes con tuberculosis pulmonar farmacorresistente. Estas cepas se analizaron mediante tipificación con oligonucleótidos de los espaciadores polimórficos de las regiones DR (spoligotyping) y la huella genética del ADN con la secuencia de inserción IS*6110*. Una parte de estas cepas se caracterizó también secuenciando los genes *rpoB* y *katG* o la región reguladora del gen *inhA*, o ambos.
RESULTADOS : La utilización combinada del spoligotyping y del análisis de los fragmentos de restricción de longitud polimórfica (RFLP) de la secuencia IS*6110* definió varios conglomerados ; el 29% de las cepas formó parte de conglomerados, lo cual indica una posible transmisión reciente. En algunos casos, pudieron confirmarse vínculos de transmisión entre las cepas de los conglomerados mediante los datos epidemiológicos y además, en la mayoría de las cepas, mediante el análisis de las mutaciones asociadas con resistencia a rifampicina, isoniacida o a ambas. La edad temprana, el sexo, la inmigración y el antecedente de tratamiento no se asociaron con la creación de conglomerados, pero la tuberculosis con multidrogorresistencia presentó mayor probabilidad de pertenecer a un conglomerado. Se encontraron cepas de la familia Beijing también en Polonia, pero con una frecuencia mucho menor que en los países circundantes.
CONCLUSIÓN : Se demostró la transmisión de cepas de *M. tuberculosis* resistentes a los medicamentos, lo cual puede contribuir a la aparición de tuberculosis farmacorresistente en Polonia.