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A Two-Step Strategy for Molecular Typing of Multidrug-Resistant *Mycobacterium tuberculosis* Clinical Isolates from Poland

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Abstract

Tuberculosis (TB) continues to be one of the most challenging public health problems in the world. An important contributor to the global burden of the disease is the emergence and spread of drug-resistant and particularly multidrug-resistant *Mycobacterium tuberculosis* strains (MDR), defined as being resistant to at least isoniazid and rifampicin. In recent years, the introduction of different DNA-based molecular typing methods has substantially improved the knowledge of the epidemiology of TB. The purpose of this study was to employ a combination of two PCR-based genotyping methods, namely spoligotyping and IS*6110*-Mtb1/Mtb2 PCR to investigate the clonal relatedness of MDR *M. tuberculosis* clinical isolates recovered from pulmonary TB patients from Poland. Among the 50 isolates examined, 28 (56%) were clustered by spoligotyping, whereas IS*6110*-Mtb1/Mtb2 PCR resulted in 16 (32%) clustered isolates. The isolates that clustered in both typing methods were assumed to be clonally related. A two-step strategy consisting of spoligotyping as a first-line test, performed on the entire pool of isolates, and IS*6110*-Mtb1/Mtb2 PCR typing as a confirmatory subtyping method, performed only within spoligotype-defined clusters, is an efficient approach for determining clonal relatedness among *M. tuberculosis* clinical isolates.

Key words: *Mycobacterium tuberculosis*, multidrug resistance, spoligotyping, IS*6110*-Mtb1/Mtb2 PCR

Introduction

Tuberculosis (TB) continues to be one of the greatest public health problems in the world. Despite significant improvements in diagnosis and treatment procedures, every year TB kills about 2 million people, while an estimated 9 million develop the disease (WHO, 2006). Of the major contributors to the global TB burden is the appearance and spread of drug-resistant (DR), and, particularly, multidrug-resistant *Mycobacterium tuberculosis* strains (MDR; defined as resistant to at least isoniazid [H] and rifampicin [R] – two major anti-TB drugs). In Poland, the phenomenon of drug resistance in TB has been closely monitored since the mid 1990s, when Poland joined the World Health Organization (WHO) and International Union Against Tuberculosis and Lung Disease (IUATLD) global project on anti-TB drug resistance surveillance, and performed in 1996 the first prospective, country-wide survey (Zwolska *et al.*, 2000). Based on the data of the 3rd national survey on TB drug resistance conducted in 2004, the number of DR- and MDR-TB cases was 246 (7.6%) and 51 (1.6%), respectively, placing Poland among the countries with relatively low DR-TB rates in the world (WHO and IUATLD, 2008).

Over the last several years, the application of different DNA-based molecular typing methods has substantially improved our knowledge of the epidemiology of TB (Mathema *et al.*, 2006). The usefulness of those methods has been demonstrated primarily as epidemiological markers to discriminate the pathogen at the genus, species, and subspecies level. The strain-level differentiation is of crucial importance for disclosing the sources of infection (exogenous *vs* endogenous acquisition), elucidating its potential routes of transmission, determining whether the infection is caused by a single

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strain or by multiple strains (homogeneous *vs* mixed infection), and whether recurrence of the disease is attributable to treatment failure, that is relapse of infection with the original strain, or infection with a new strain of *M. tuberculosis* (endogenous reactivation *vs* exogenous reinfection). Furthermore, strain typing can be used to study the molecular mechanisms that mediate host-pathogen interactions or to identify the genotype-specific differences in the phenotypic characteristics, such as virulence, sensitivity to antimicrobial agents, organ tropism, transmissibility *etc.* (Mathema *et al*., 2006).

The purpose of this study was to employ a combination of two PCR-based genotyping methods, namely spoligotyping and IS*6110*-Mtb1/Mtb2 PCR to investigate the clonal relatedness of MDR *M. tuberculosis* clinical isolates recovered from pulmonary TB patients (PTB) from Poland.

Experimental

Material and Methods

Patients and bacterial strains. The study included a total of 50 MDR *M. tuberculosis* isolates recovered from 46 unrelated adult PTB patients from Poland (two isolates were obtained from each of four patients). The MDR-TB cases included in this study represented all bacteriologically-confirmed MDR-TB cases reported in Poland throughout 2004. Patients (40 men and 6 women; age range, 31 to 79 years; median age, 50.5 years) were recruited in 20 TB clinics in 11 Polish voivodeships: kujawsko-pomorskie (8 patients), mazowieckie (8), dolnośląskie (7), lubelskie (7), śląskie (5), małopolskie (4), łódzkie (2), pomorskie (2), podlaskie (1), świętokrzyskie (1) zachodnio-pomorskie (1), during a 1-year period (*i.e.* from 1 January 2004 to 31 December 2004). Primary isolation, species identification, and drug susceptibility testing (DST) were done at the regional mycobacteriology laboratories. The isolates were subcultured and sent to the National TB Reference Laboratory (NTRL) at the National Tuberculosis and Lung Diseases Research Institute in Warsaw, where confirmatory identification and DST were performed. Susceptibility testing to the first-line drugs was carried out according to the 1% proportion method, in Löwenstein-Jensen (L-J) medium, with the following critical concentrations: isoniazid [H] (0.2 μg/ml), rifampicin [R] (40 μg/ml), streptomycin [S] (4 μg/ml), and ethambutol [E] (2 μg/ml). Patient demographic and clinical characteristics were collected from the hospitalization records.

Apart from the patient clinical isolates, two reference strains were used for spoligotyping, *i.e.*: *M. tuberculosis* H_{37}Rv and *M. bovis* BCG.

DNA isolation. Genomic DNA was obtained from *M. tuberculosis* colonies on L-J slants by the cetyltrimethyl-ammonium bromide (CTAB) method (van Embden *et al*., 1993).

Genotyping methods. Spoligotyping was performed with a commercially available kit (Isogen Bioscience BV, Maarssen, The Netherlands) according to the instructions provided by the manufacturer and as described previously (Kamerbeek *et al*., 1997). The IS*6110*-Mtb1/Mtb2 PCR typing was performed according to the methodology described earlier (Kotłowski *et al*., 2004). Briefly, 2 μl of *Pvu*II-restricted genomic DNA was used for two PCR assays, using a combination of primers IS1 and IS2, binding to the inverted repeats flanking IS*6110*, and either Mtb1 or Mtb2 primers, targeted to the repeated GC-rich sequences. The PCR products were resolved electrophoretically on 2% agarose gels and visualized under UV light after ethidium bromide staining.

Analysis of genotyping results. The spoligotyping results were read independently by two observers, expressed as an octal code, entered in an Excel spreadsheet file and compared to the international spoligotype database (SpolDB4) at the Pasteur Institute of Guadeloupe (www.pasteur-guadeloupe.fr/tb/spoldb4), which at the time of matching analysis contained 39,295 patterns split into 1,939 shared types and 3,370 orphan profiles from 122 countries (Brudey *et al*., 2006b).

A spoligotype cluster (the term „cluster" always referred to a group of at least two isolates that derived from different patients) was defined as two or more isolates exhibiting 100% identity of their spoligotype patterns, whereas those non-clustered were referred to as unique. The isolates whose spoligotype patterns were already recorded in the database, were assigned "shared types", whereas those of spoligotypes identified for the first time, were designated either as "new shared types" (if 2 or more) or as "orphans" (if occurred only once).

The SpolDB4 was also used to assign genotype families to the spoligotypes obtained. The spoligotypes absent in the SpolDB4 database, or of unknown family ("U"), were identified at a family level by the "SpotClust" program, which implements a mixture model built on the SpolDB3 database (Vitol *et al.*, 2006; http://cgi2.cs.rpi.edu/~bennek/SPOTCLUST.html).

In IS*6110*-Mtb1/Mtb2 PCR typing, the patterns obtained in each of the two PCR assays, dependently on the primer combination (Mtb1-IS1-IS2 or Mtb2-IS1-IS2), were analysed separately with LabWorks 4.5 software (UVP, Inc., Upland, CA, USA) and by visual inspection. The molecular sizes of the PCR fragments were calculated using a 50-bp DNA ladder (Promega) as a molecular weight standard. Strains whose band patterns were identical (with a 5.0% band size tolerance) were defined as indistinguishable. However, isolates

with less than 5 bands were defined as different, even if there was a single-band difference. Only if indistinguishable upon IS*6110*-Mtb1 and IS*6110*-Mtb2 PCR typing, the strains were considered clustered. The isolates were assumed clonally related only if clustered by both genotyping methods.

A spoligotype-based dendrogram was generated with BioNumerics software (Applied Maths, Kortrijk, Belgium), whereas dendrograms based on IS*6110*-Mtb1/Mtb2 PCR analyses were drawn using the GelQuest/ClusterVis (Sequentix, Klein Raden, Germany). Similarity between fingerprints was each time calculated with the Dice's coefficient, and clustering was performed using UPGMA (Unweighted Pair Group Method with Arithmetic Averages) algorithm.

Results

Spoligotyping of the analysed *M. tuberculosis* isolates produced a total of 27 different spoligotype patterns. Of these, 7 patterns defined as many clusters, including 28 (56%) isolates from 26 (56.5%) patients (the size of the clusters ranged from 2 to 8 isolates). The remaining 20 spoligotypes were unique, being found in 22 (44%) isolates from 20 (43.5%) patients (in two cases, both isolates from the same patient had identical spoligotypes).

By comparison with the international spoligotype database (SpolDB4), 40 isolates from 37 (80.4%) patients harboured spoligotypes that had already been reported. For those isolates, specific international

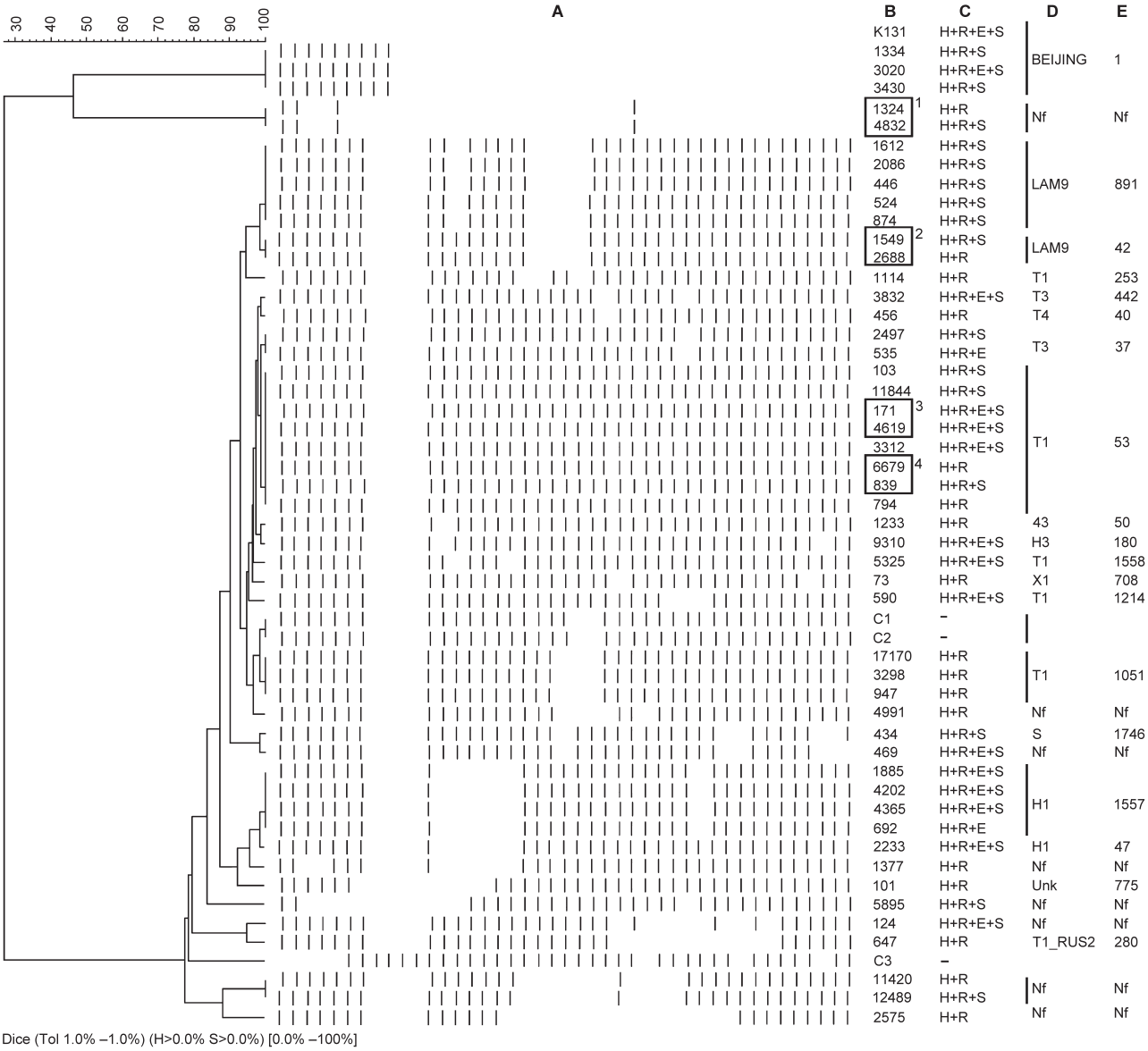

Fig. 1. Spoligotype dendrogram drawn for the 50 *M. tuberculosis* isolates used in this study.

A, spoligotype hybridization pattern; B, isolate number; C, drug resistance profile; D, molecular family; E, shared type (ST) designation, according to the SpolDB4; H, isoniazid; R, rifampicin; E, ethambutol; S, streptomycin; C1-C3, reference strains: *M. tuberculosis* $H_{37}Rv$ (C1, C2), *M. bovis* BCG (C3); Nf – not found (in the SpolDB4); Unk, family unknown. Note the double isolates from the same patient were put in frames (1–4).

Table I
Spoligotypes unrecorded in the SpolDB4 and their family assignment by SpotClust analysis

	Spoligotype[a]			Family[b]	P[c]	N[d]
		Binary	Octal			
1.	A	■■■■■■■■■■■■■□□□□■□□□□□□□■■■■■■■□□□□■■■■■■■	777741003760771	LAM9	0.94	2 (11420; 12489)
2.	B	□□□□□□□□□□□□□□□■□□□□□□□□□□□□□□□□□□□□□■□□■■	000002000000111	LAM7	0.69	2 (1324; 4832)*
3.	C	□□□■■■■■□□■■■■■■■■■■■□■■■■■■■■■■□□□□■■■■■■■	076377737760771	S	0.78	1 (469)
4.	D	■■■■■■■■■□□□□□□□□□□□□□□□□■■■■■■□□□□■■■■■■■	777000001760771	T4	0.97	1 (2575)
5.	E	■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■□□□□■■■□□■■	777777774020711	H1	0.99	1 (1377)
6.	F	■■■■■■■■■■■□■■■■■■■■■■■■■□□□□□□□□□□□□■■	777737777600011	T2	0.91	1 (5895)
7.	G	■■■■■■□■□■□□■□□□□□■□■■■■■■■■■■■■■□□□□■■■■■■■	772202777760771	S	0.95	1 (124)
8.	H	■■■■■■■■■■■■■■■□■■□□□□■■■■■■■■■■□□□□■■■■■■■	777773037760771	H_{37}Rv	0.98	1 (4991)
9.	775	■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□□□□□■■■■■■	777777777000371	H3	0.69	1 (101)

[a] Spoligotype, A-H, arbitrary spoligotype designation;
[b] SpotClust-assigned family;
[c] P, probability of the spoligotype pattern to belong to the family;
[d] N, number of isolates; in brackets are given isolate numbers; an asterisk indicates isolates from the same patient.

shared-type (ST) designations could be assigned (Fig. 1). The most prevalent among the 19 SpolDB4-defined spoligotypes found in this study were: ST53, ST891, ST1, ST1557, and ST1051, embracing 8, 5, 4, 4, and 3 isolates, respectively.

Nine (19.6%) patients had isolates whose spoligotypes did not match any existing ST. Two of those spoligotypes were defined as new STs, as they were common in 2 isolates from one patient or 2 isolates from two different patients. Those new STs were designated "A", and "B", respectively. Another 6 spoligotypes (designated "C"-"H") that were unrecorded in the SpolDB4 occurred only once, and were thus defined as orphan types (Table I).

Analysis of the geographical distribution of the spoligotypes found in this study revealed that 16 (59.2%) spoligotypes, representative of 37 (74%) isolates, had been observed in Poland previously (in past studies).

The family assignment, based upon SpolDB4 and SpotClust analysis revealed that 42% of the isolates belonged to the T family, 22% to the Latin American Mediterranean (LAM) family, and 18% to the Haarlem (H) family. Four other minor families, that is Beijing, S, X, and H_{37}Rv family accounted for 8%, 6%, 2%, and 2% of all isolates, respectively.

With the aim of further differentiation, all patient isolates were subjected to IS*6110*-Mtb1/Mtb2 PCR typing. The results showed that 10 (35.7%) isolates being part of spoligotype clusters produced unique banding patterns. Thus, one spoligotype cluster (ST37) was ruled out. Another cluster (ST53), containing 8 isolates from 6 patients was split into two subgroups; the first was composed of two isolates from the same patient, while the second was comprised of 3 isolates, of which 2 belonged to one patient. (The latter group met the criterion of a cluster). The IS*6110*-Mtb1/Mtb2

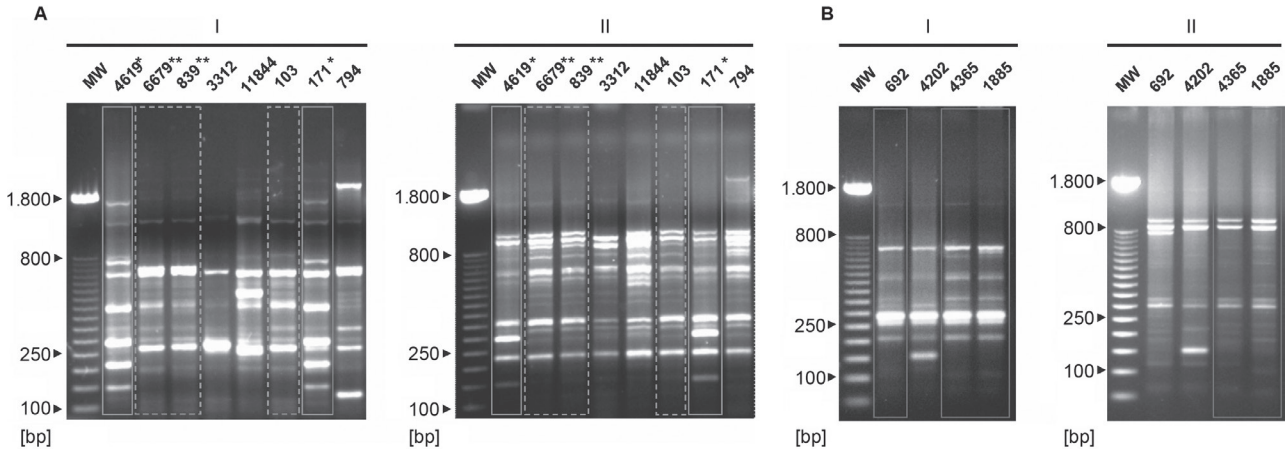

Fig. 2. IS*6110*-Mtb1/Mtb2 PCR patterns for two selected spoligotype-defined clusters: ST53 (A) and ST1557 (B).
I and II designate two PCR assays with IS1-IS2-Mtb1 (I) and IS1-IS2-Mtb2 (II) primer combination; MW, molecular weight marker. Numbers above the gel lines are isolate numbers. Asterisks (*; **) indicate isolates from the same patient. Note that isolates with identical DNA fingerprints were put in frames.

Table II
Clustering results of the two genotyping methods used in this study

Method	No of clustered isolates (%)	No of clusters	Size of cluster	HGDI
Spoligotyping	28 (56)	7	2–8	0.9535
IS*6110*-Mtb1 PCR	17 (34)	6	2–4	0.9837
IS*6110*-Mtb2 PCR	16 (32)	6	2–4	0.9853
IS*6110*-Mtb1/Mtb2 PCR	16 (32)	6	2–4	0.9853

PCR patterns of the remaining three isolates from the spoligotype cluster ST53 were unique (Fig. 2A). In three clusters, the number of clustered isolates dropped from 5 to 4 (ST891), and from 4 to 2 (ST1, ST1557). Only 2 spoligotype clusters (ST1051, "A"), containing 3 and 2 isolates respectively, were identical with IS*6110*-Mtb1/Mtb2 PCR.

For the 22 isolates with unique spoligotypes, the patterns generated by IS*6110*-Mtb1/Mtb2 PCR were also found unique.

In IS*6110*-Mtb1/Mtb2 PCR typing, the clustering of the patterns generated from each of the two PCR assays, with either Mtb1-IS1-IS2 or Mtb2-IS1-IS2 primer combination was highly concordant (Fig. 3). The only difference was in the subtyping within the ST1557 cluster. Three or two isolates clustered when IS*6110*-Mtb1- or -Mtb2 PCR was performed (Fig. 2B).

In all four cases, double isolates from the same patient were identical with respect to their spoligotypes and IS*6110*-Mtb1/Mtb2 PCR patterns.

Clustering results obtained with each of the two typing methods are compared in Table II.

Overall, a combined analysis of spoligotyping and IS*6110*-Mtb1/Mtb2 PCR resulted in 6 clusters comprising of 16 isolates (32% of all isolates) from 15 patients (32.6% of all patients). The isolates within those clusters were assumed to be clonally related.

Discussion

Although *M. tuberculosis* constitutes a remarkably genetically homogeneous species, various repetitive DNA elements have been found in the *M. tuberculosis* genome that contribute to strain variation, thus providing excellent targets for molecular typing methods. The method most widely used for *M. tuberculosis* strain differentiation, considered the "gold standard" in the molecular epidemiology of TB, has been the IS*6110*-based restriction fragment length polymorphism (RFLP) analysis, which detects, through Southern blotting, a specific repetitive element insertion sequence IS*6110*, whose number of copies and distribution throughout the mycobacterial chromosome varies between the strains (Thierry *et al*., 1990; van Embden *et al*., 1993). Other repetitive element-based DNA fingerprinting techniques include spoligotyping, which relies on determining the presence or absence of unique spacer sequences interspersing the direct repeats (DRs) within the DR locus of the *M. tuberculosis* chromosome (Kamerbeek, *et al*., 1997), DRE-(double-repetitive-element) PCR, based on the detection of inter-IS*6110*-PGRS (polymorphic GC-rich sequence) polymorphism (Friedman *et al*., 1995) or more recently MIRU-VNTR (mycobacterial interspersed repetitive unit-variable number of tandem repeats) typing, which targets the polymorphism of different tandem DNA repeats scattered in various intergenic loci in the mycobacterial genome (Supply *et al*., 2000; Supply *et al*., 2001).

In the molecular epidemiology studies of TB, the choice of the most appropriate typing method is of the utmost importance. First of all, such a method should have high enough discriminatory power to clearly discriminate among unrelated isolates of *M. tuberculosis* and at the same time to establish a clonal relationship between related strains. The higher the discriminatory power of the method, the greater the probability that the isolates within the clusters are truly related. The use of a poorly discriminative technique will overestimate the number of clustered isolates, thus leading to a false reconstruction of the transmission patterns within the population studied. Spoligotyping, which has important advantages of technical simplicity, robustness, time- and cost-effectiveness, portability and high reproducibility, has been repeatedly shown to overestimate clustering (Kremer *et al*., 1999; *Gori et al*., 2005). For instance, in a study of Gori *et al*. (2005), the number of isolates with identical spoligotypes was twice the number of isolates with identical IS*6110*-RFLP patterns. The low discriminatory capacity of the spoligotyping precludes its use as a sole genotyping method for epidemiological studies. However, spoligotyping has been found highly efficient when used in association with a second-line test, such as IS*6110*-RFLP (Goyal *et al*., 1997; Gori *et al*., 2005) or a PCR-based method, such as DRE-PCR (Sola *et al*., 1998) or MIRU-VNTR typing (Sola *et al*., 2003; Oelemann *et al*., 2007).

Although the IS*6110*-RFLP is the reference technique for genotyping *M. tuberculosis*, because of its high discriminatory index, several limitations and drawbacks have been demonstrated for this method. First, it requires large amount of extracted and highly purified DNA, necessitating lengthy subculturing of *M. tuberculosis*. Second, it provides insufficient discrimination among isolates carrying six or fewer copies of IS*6110* (Bauer *et al*., 1999; Kremer *et al*., 1999). Finally, IS*6110*-RFLP suffers from the difficulty of analysing and

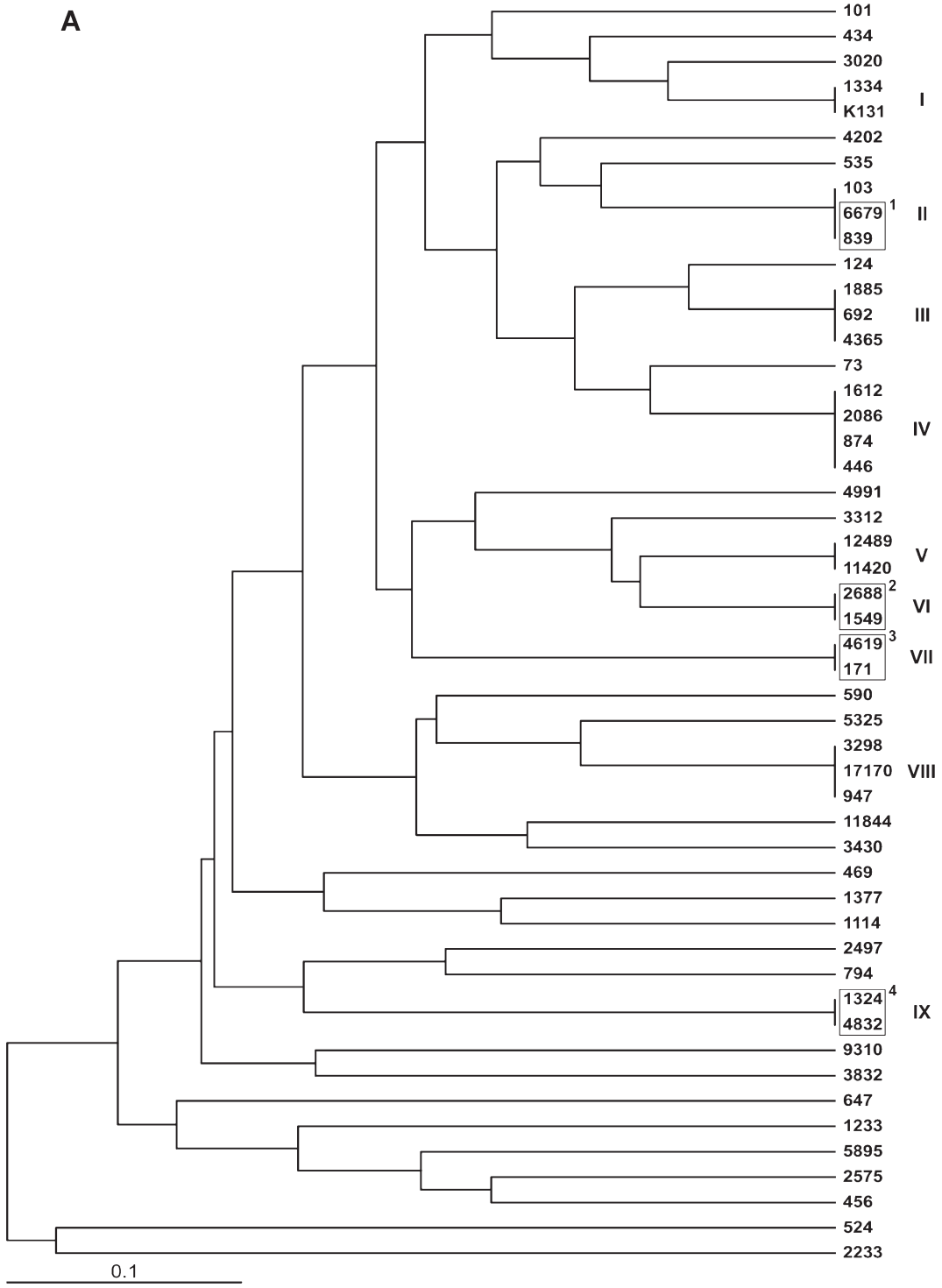

Fig. 3A.

interpreting of complex banding patterns. Databasing and interlaboratory comparison of the IS*6110*-RFLP profiles are technically demanding, requiring specialized software and expertise in its operation. Consequently, there is still a need for novel genetic markers that would unambiguously distinguish genetically related from genetically distinct (unrelated) isolates. A strategy integrating two rapid PCR-based methods has been suggested as a potential alternative to IS*6110*-RFLP for genotyping of *M. tuberculosis* (Goguet de la Salmonière *et al*., 1997). The best results were achieved when combining spoligotyping with MIRU-VNTR typing. The discrimination ability of such a system was found to be higher that that of IS*6110*-RFLP analysis (Oelemann *et al*., 2007; Allix-Beguec *et al*., 2008).

In this study, we evaluated the applicability of another two-step strategy in typing *M. tuberculosis* clinical isolates. The strategy involved two PCR-based methods:

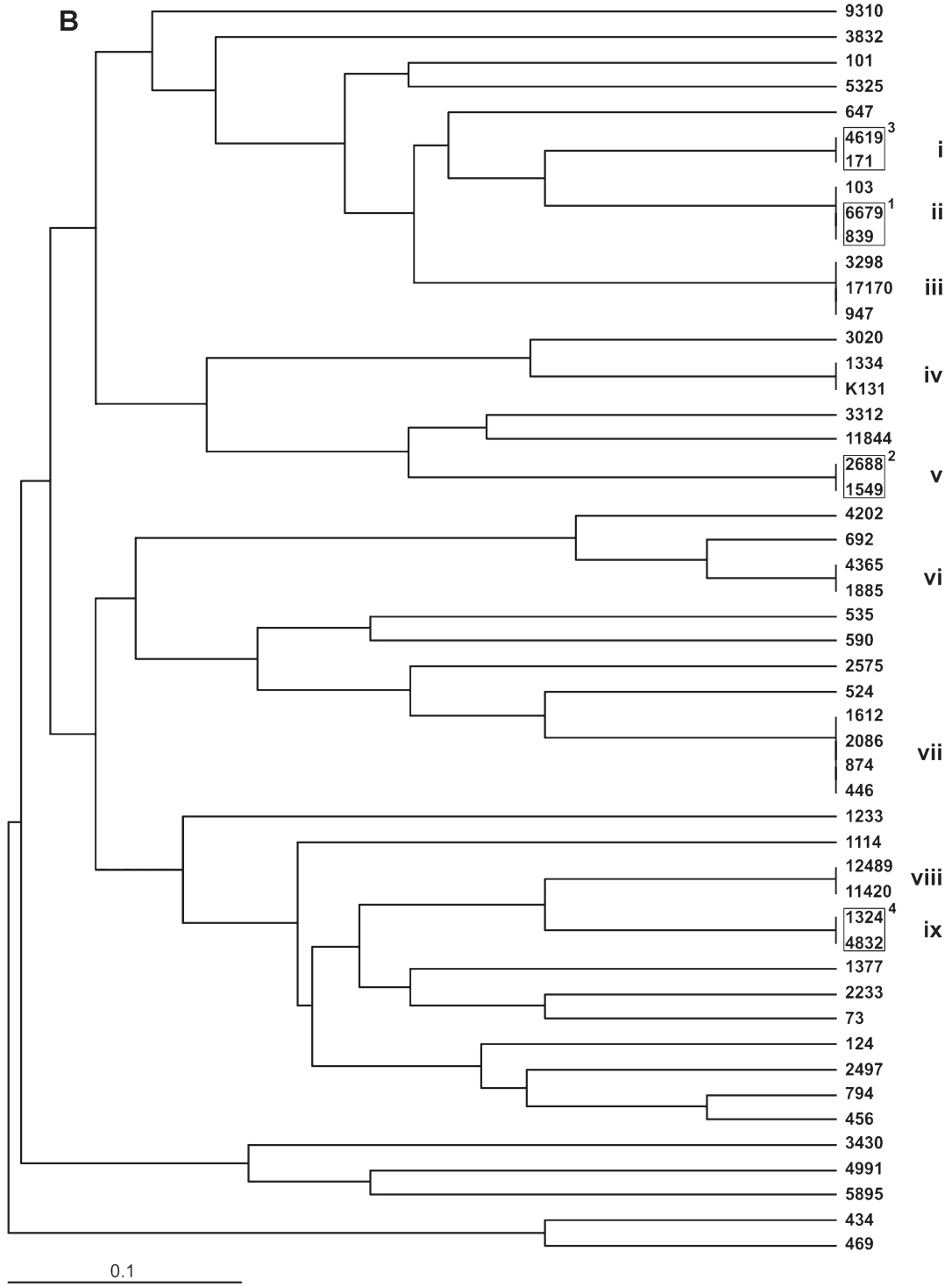

Fig. 3. Dendrogram generated for the 50 *M. tuberculosis* isolates, based on computer-assisted comparison of DNA fingerprints obtained in two PCR assays with IS1-IS2-Mtb1 (A) and IS1-IS2-Mtb2 (B) primer combination.

Roman numerals (I–IX; i–ix) indicate groups of 2 or more isolates harbouring identical banding patterns. Only groups indicated I–V, and VIII, as well as groups indicated ii–iv, and vi–viii are clusters. Note the double isolates from the same patient were put in frames (1–4).

spoligotyping and IS*6110*-Mtb1/Mtb2 PCR. The latter method has been proposed as a new marker system for strain differentiation of tubercle bacilli by Kotłowski *et al.* (2004). The strength of this method is that it is fast, fairly robust, and easy to perform. More importantly, it possesses high discriminatory power, higher that that of spoligotyping and MIRU-VNTR typing, and comparable to that of IS*6110*-RFLP, as indicated in preliminary studies (Sajduda *et al.*, 2006). The superiority of the IS*6110*-Mtb1/Mtb2 PCR over spoligotyping, in terms of discriminatory potential, was further demonstrated in two studies by Augustynowicz-Kopeć *et al.* (2007;

2008b). This was also confirmed in the present study. The clustering rate for spoligotyping and IS*6110*-Mtb1/Mtb2 PCR was 56% and 32%, respectively. It should also be stressed here that in all studies utilizing IS*6110*-Mtb1/Mtb2 PCR typing, carried out so far (including the present one), isolates with unique spoligotypes also bore unique IS*6110*-Mtb1/Mtb2 PCR patterns. This observation justifies the performance of IS*6110*-Mtb1/Mtb2 PCR typing only within spoligotype clusters (*i.e.* for differentiation of isolates belonging to spoligotype clusters). A two-step protocol, in which spoligotyping is used as a preliminary screening test, and is followed by another typing method, of greater discriminatory capacity, performed on isolates with the same spoligotypes, has already been deployed by other investigators (Goguet de la Salmonière *et al*., 1997; Sola *et al*., 1998; Brudey *et al*., 2006a).

Accurate assessment of genetic relatedness between *M. tuberculosis* isolates depends on cluster definition. Most studies, including the current one, define "clustered isolates" as those sharing an identical DNA fingerprint. In general, the more lenient the criteria, the higher chance of finding clusters, but the lower the likelihood that a cluster represents clonally related isolates, being part of the same chain of TB transmission (Glynn *et al*., 1999). However, in certain studies a single band difference between two DNA fingerprints is an allowable criterion to define a cluster (Goyal *et al*., 1997; Lari *et al*., 2007). This tolerance is supported by the documented cases of TB transmission between patients whose isolates had similar but not identical genotypic patterns (Ijaz *et al*., 2002). Based on the results from this study, the situation described above might have occurred only in one case. Among four isolates belonging to the ST1557 spoligotype cluster, two isolates had identical IS*6110*-Mtb1/Mtb2 PCR patterns, whereas one isolate differed from them by a single band, in only one PCR assay (with Mtb2-IS1-IS2 primer set) (Fig. 2B). Hence, in this particular situation, not two but three isolates could make up the final cluster. In all the remaining cases, the patterns resulted from subtyping of spoligotype clusters were either identical or differed by at least two bands.

Regarding the phylogenetic structure of the *M. tuberculosis* population studied, inferred by confronting the spoligotyping results with the international spoligotype database (SpolDB4), three families: T, LAM, and Haarlem were shown predominant, accommodating 82% of the patients' isolates. Overall, the genetic structure of Polish MDR *M. tuberculosis* isolates was characteristic of a European country (Brudey *et al*., 2006a; David *et al*., 2007; Lari *et al*., 2007). It was also similar to what had been reported for DR-TB in Poland in the past years (Sajduda *et al*., 2004; Augustynowicz-Kopeć *et al*., 2008a). The finding that 74% of the isolates from the MDR-TB cases studied displayed spoligotypes that had previously been shown in Poland may suggest that strains with these genotypes have been circulating in Poland for a long time and have been actively transmitted within the country. The presence of unique spoligotypes, hitherto unreported in the spoligotype database may suggest their specificity to the study setting.

In conclusion, this study demonstrates the usefulness of a two-step strategy consisting of spoligotyping as a first-line test, performed on the entire pool of isolates, and IS*6110*-Mtb1/Mtb2 PCR typing as a confirmatory subtyping method, performed only within spoligotype-defined clusters, for determining the clonal relatedness among *M. tuberculosis* clinical isolates.

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