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Mycobacterium tuberculosis – the 10 years of epidemiological and diagnostics studies

Abstract: Tuberculosis (TB) is the main bacterial pathogen that causes more deaths than AIDS, malaria and all infectious diseases. The unusual long doubling time (about 24h), highly hydrophobic cell envelope resistant to chemical lysis was the reason to delay the molecular study of this bacteria. Fifteen years ago, we did not have any molecular tools and methods for genetic manipulation or isolation and analysis of intracellular protein and nucleic acids. Today we have many useful shuttle or integration vectors for basic study of mycobacteria. The full sequence of M. tuberculosis genome is already known. At the present time the diagnosis of tuberculosis is supported with fast-culture system BACTEC and molecular techniques based on PCR and DNA hybridization. The mechanisms of resistance to antituberculosis drugs were described, and first identification of resistance profile is available by using PCR and sequencing or real time PCR methods. In our group in the Center for Microbiology and Virology Polish Academy of Sciences and in the Dept. of Genetics of Microorganisms, University Łódź we have characterized new insertion sequences from *M. tuberculosis* complex-IS990 and IS1607. In diagnostic studies we have proposed the DIG-PCR ELISA assay as a reliable, specific and sensitive test to identify M. tuberculosis directly in clinical samples. We have performed wide epidemiological studies of *M. tuberculosis* strains isolated from Polish TB patients including drug – and multidrug – resistant strains. Finally we identified the most frequently present mutations responsible for drug resistance of polish clinical isolates of *M. tuberculosis*.

Key words: *Mycobacterium tuberculosis*, insertion sequences, PCR, epidemiology, diagnosis.

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1. Introduction

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium microti*, *Mycobacterium canetti* and is a persistent problem in the developing world. The World Health Organization estimates that approximately one-third of the world's population is infected with tuberculosis and in this decade alone, almost 90 million people will contact tuberculosis (KOCHI 1999). During the same time period, approximately 3 million people die annually from this disease, resulting in more deaths than are caused by any other single pathogen (CDC, 1999).

M. tuberculosis has posed a formidable challenge to biomedical researchers as a result of its long generation time, fastidious growth requirements, and high risk of contagion. In the last decade, the development of efficient vectors and allelic exchange system has rendered the tubercle bacillus more amenable to molecular genetic analysis and this has greatly facilitated studies of its biochemistry, physiology, and pathogenicity (BARADOV et al. 1997; PELICIC et al. 1997). The availability of the complete genome sequence and its detailed bioinformatics analysis has provided us with a wealth of new information, knowledge, and understanding of the biology of this major human pathogen (COLE et al. 1998).

2. *Mycobacterium tuberculosis* Insertion Sequences

The only DNA mobile elements of *M. tuberculosis* (excluding bacteriophages) are insertion sequences. The best-known insertion sequence of *M. tuberculosis* is IS*6110*, insertion element present in different number of copies in various loci in the genomes of species belonging to the *M. tuberculosis* complex (KENT et al. 1995; MCHUGH et al. 1997). However, it was reported that some *M. tuberculosis* strains exist that do not posses the IS*6110* insertion sequence in their genomes (SAJDUDA et al. 1998). The IS*6110* insertion sequence has previously been reported to be specific for the *M. tuberculosis* complex, but IS*6110*-like sequences are present in MOTT (SJÖBRING et al. 1990). This multicopy element is widely used both for epidemiological and diagnostic studies. The first mycobacterial insertion sequence was discovered in 1987, until today 46 IS-like elements are known. The sequencing of *M. tuberculosis* genome revealed 25 new IS-like sequences. Of the 46 mycobacterial ISs described, only 6 have been shown to be active and able to transpose from one site on a DNA molecule to another. Most of these 46 ISs are restricted to very limited host range, making them

a good tool for the molecular typing of mycobacteria (e.g. IS990, IS1081, and IS1607).

We have characterized a new insertion sequence from *M. tuberculosis*, it is a member of the IS3 family and is related to IS*6110* (Fig. 1) (DZIADEK et al. 1998). A characteristic of these elements is the involvement of ribosomal frame shifting in the generation of functional transposase by fusion of the ORFa and ORFb polypeptides. The two ORFs in IS*990* have been shown to display significant homology to IS3 ORFa and ORFb sequences. The potential frame shift region overlaps exactly with the same region of the IS*6110* element. The nucleotide homology of the region including 30 nucleotides overlap between IS6110 and IS990 is 70%. In contrast to IS*6110*, IS*990* is present as only a single copy in all strains of *M. tuberculosis* – complex that have been tested so far. *Mycobacterium bovis*, *Mycobacterium bovis* BCG, *M. microti* and *M. africanum* strains revealed the presence of IS*990* insertion sequence by hybridization and PCR-based analysis. To determine the host range of IS990, the chromosomal DNA from different species of mycobacteria was extracted and analyzed by Southern hybridization using fragment of IS*990* as the probe. Fourteen different strains of *Mycobacterium avium*, two *M. simiae* strains, two *M. lufu* strains, two *M. malmoense* strains, *M. intracellularae*, *M. gordonae*, *M. fortuitum*, *M. scrofulaceum* and *M. paratuberculosis* were tested. None of these showed hybridization signal when used with the investigated probe (Fig. 2). These data were confirmed by PCR- based analysis (the expected amplification product was observed only in the case of *Mycobacterium tuberculosis* complex bacteria). By the end of 2000, 350 clinical isolates of *M. tuberculosis* have been examined; all *M. tuberculosis* strains contain IS*990* DNA. The presence of IS990 as a single copy element in *M. tuberculosis* strains strongly suggests its possible application in diagnosis of *M. tuberculosis* infections.

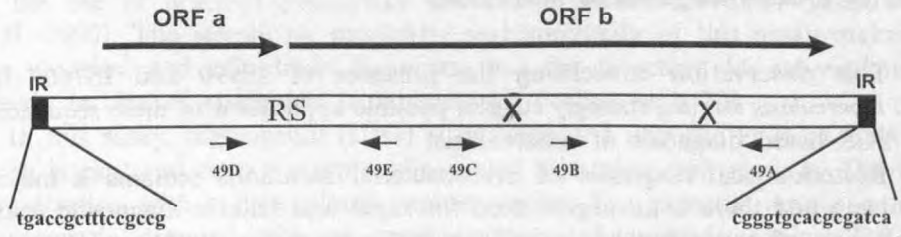

Fig. 1. The composition of IS990 belonging to the IS3 family. IS990 is bordered by 16 bp inverted repeats (IRs). The ribosome slippage region is marked with RS. The mutations in ORFb are indicated by Xs. Primers used for amplification of IS990 fragments are shown as arrows and designated as 49 A-E. The nucleotide positions of the start and end of IS990 are correlated with Y49 cosmid

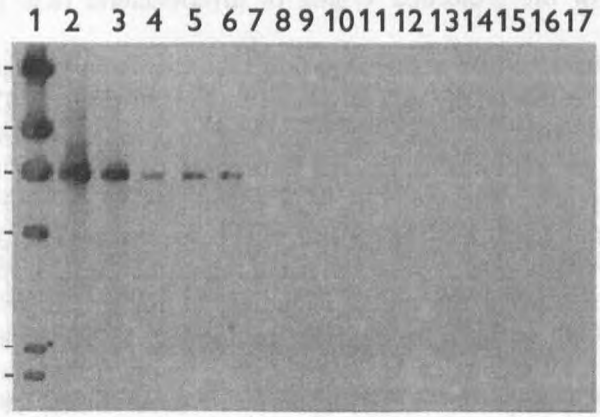

Fig. 2. The species – specificity analysis of IS990 by Southern hybridisation with PvuII-digested DNAs from different mycobacterial species. *M. bovis* BCG (3–4), *M. bovis* (5–6), *M. scrofulaceum* (7), *M. fortuitum* (8), *M. gordonae* (9), *M. malmoense* (10–11), *M. lufu* (12–13), *M. simiae* like A (14), *M. simiae* like B (15), *M. intracellulare* (16), *M. avium* (17). Lane 1 represents the molecular mass marker (bacteriophage λ DNA restricted by *Hind*III) and lane 2 was loaded with the DNA of *M. tuberculosis* 370 (positive control)

We also described insertion sequence IS1607 – a new *M. tuberculosis* complex sequence – related element (DZIADEK et al. 2000). IS*1607* was also present as a single copy in *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG strains. This sequence existed in *M. africanum* and *M. microti*, but was not present in 69 strains of 19 atypical mycobacterial species analyzed by using polymerase chain reaction (PCR) or Southern hybridization assay.

2.1. PCR-based diagnosis of tuberculosis

This observation concerning the presence of IS*990* and IS*1607* in *M. tuberculosis* strains, strongly suggest possible application of these sequences in PCR-based diagnosis of tuberculosis.

Bacteriological diagnosis of mycobacterial infections remains a major problem and there is an urgent need for rapid and reliable diagnostic tests. Culturing of organisms has specificity that approaches 100% and permits susceptibility testing of the isolates, but the slow growth of most pathogenic mycobacteria (4 to 6 weeks) results in delays in diagnosis (SJÖBRING et al. 1990; SHAWAR et al. 1993). Identification of acid-fast bacilli stained smears may provide rapid diagnosis, but this method does not allow identification at the species level and requires a relatively large number of bacteria ($> 10^4$

cells/ml) present in a sample (KOLK et al. 1992). Serological techniques may be useful in some clinical settings, but this approach is limited generally due to poor sensitivity and/or specificity. The DNA or RNA hybridization tests with labeled specific probes are not sensitive enough to be used for clinical specimens without prior culturing. The lack of simple, rapid, and reliable tests that can specifically detect *M. tuberculosis* in clinical specimens poses enormous problems for both individual patient management and implementation of appropriate infection control and public health measures. In the last decade PCR has set to be a useful tool for the direct identification of mycobacteria in either cultured strains or uncultured clinical samples. PCR tests have been developed for diagnosis of tuberculosis (NOLTE et al. 1993). Commercially available tests are however too expensive for regular use in many regions where TB is most common. "In-house" PCR-based tests have been developed, many of which utilize mycobacterial insertion sequence IS*6110* as target for the PCR (HUANG et al. 1996). However, IS*6110* is not ideal as a target, since its copy number is variable and strains of *M. tuberculosis* have been identified that do not contain IS*6110* (MCFADDEN, SAJDUDA 1996; SAJDUDA et al. 1998). Therefore, we have developed PCR-ELISA test that utilizes another *M. tuberculosis*-complex specific insertion sequence, IS*990*, as target (BORUŃ et al. 2001). We aimed to develop a simple and sensitive test that would be applicable in most clinical laboratories. We for that reason developed a simple preparation technique, followed by DNA amplification and detection of PCR product by capture plate hybridization and enzyme-linked immunoassay. We have used the *M. tuberculosis* – complex insertion sequence IS*990* as target and compared results with a similar PCR, which utilizes IS*6110* as target (THIERRY et al. 1990; DZIADEK et al. 1998). False-positive reactions that may be caused by previous amplicon contamination were prevented by the use of uracil-N-glycosylase and dUTP instead of dTTP (LONGO et al. 1990). The simplicity, sensitivity and specificity of this assay makes it a practical and affordable diagnostic test for detecting *M. tuberculosis* directly in clinical specimens in laboratories dealing with TB.

In this assay, digoxigenin (DIG) is incorporated into the PCR product, which is captured onto a streptavidin-coated microtiter well plate by DNA hybridization with a biotinylated capture probe. The captured amplicon is subsequently detected with an enzyme-conjugated antibody against DIG and a chromogenic substrate and is quantitated with an ELISA plate reader (Fig. 3). The sensitivity of DIG-PCR ELISA was determined by using serially diluted *M. tuberculosis* Mt14323 genomic DNA and then detection by using this test. The ELISA could detect 5 fg of genomic DNA, an

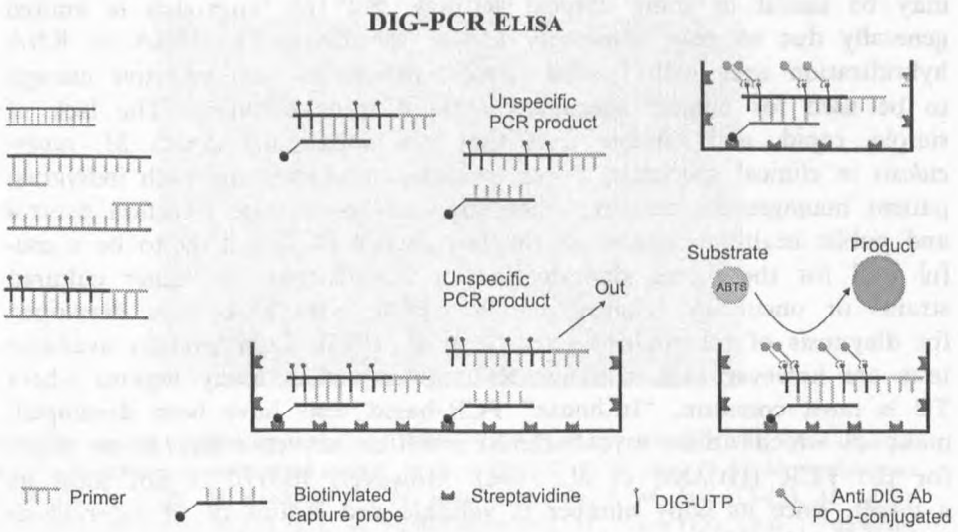

Fig. 3. DIG-PCR ELISA assay, see text for description

equivalent to approximately one mycobacterial genome. Thus, the ELISA system was shown to be at least twice more sensitive than electrophoresis through a polyacrylamide gel and then ethidium bromide staining of PCR products. Two hundred sixty five clinical samples obtained from patients with confirmed and suspected tuberculosis during 1997–1999 were analyzed by DIG-PCR ELISA (Fig. 4). These specimens were tested in parallel by conventional culture on Löwenstein-Jensen slants, a radiometric culture system (BACTEC), and DIG-PCR ELISA. For 255 samples a complete agreement between the results of all three *M. tuberculosis* detection methods was observed. 123 samples were positive (culture, BACTEC and DIG-PCR ELISA positive) and 132 were negative. Six of the remaining ten samples (4 sputa, 1 bronchoalveolar lavage and 1 stomach lavage) did not produce an *in vitro* culture (those samples were smear negative, and BACTEC negative) but were found to be positive for *M. tuberculosis* DNA in DIG-PCR ELISA. Four samples (2 sputa, 1 bronchoalveolar lavage and 1 urine) were culture and BACTEC positive (two samples were smear positive, and two were smear negative) but negative in DIG-PCR ELISA. To account for the anomalous results the clinical histories of the patients were reviewed. Six specimens that were culture – negative but PCR – positive were from patients who had clinical manifestation of TB or were suspected of having a TB reactivation and were under antituberculosis

therapy. It was previously reported that the specimens could remain PCR positive for several weeks after the initiation of effective treatment and after the time that cultures become negative (HERRERA, SEGOVIA 1996). Four PCR – negative but culture positive specimens were found. A possible explanation could be the presence of inhibitory substances in these materials (2 sputa, 1 bronchoalveolar lavage, and 1 urine), that were not entirely removed by the purification process (we tested these materials for inhibition using *M. tuberculosis* Mt14232 DNA as a template (100 ng DNA) – for 1 sputa and 1 urine PCR was also negative), or lack of sensitivity of the PCR compared to culture.

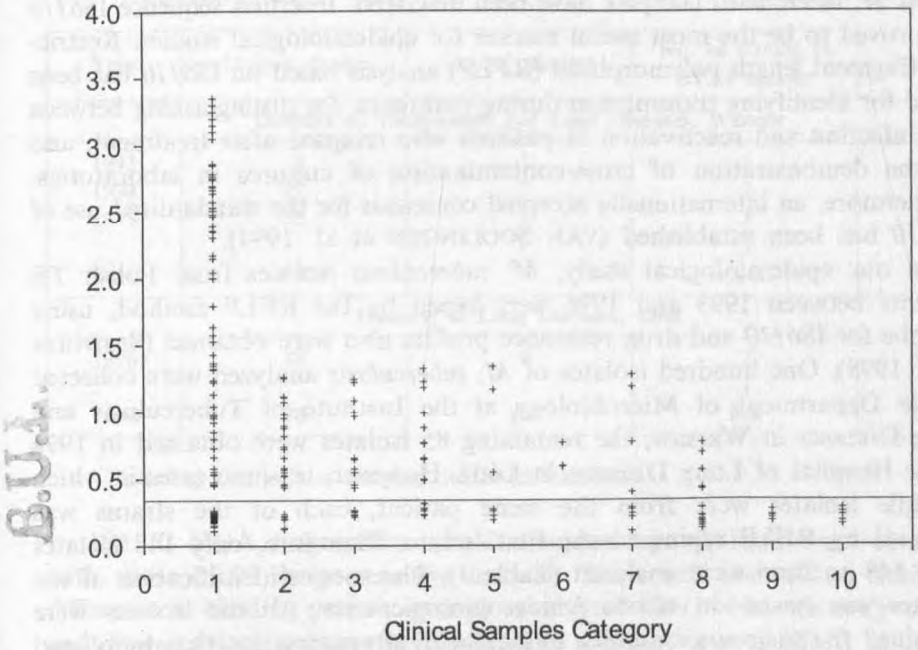

Fig. 4. Results of DIG-PCR ELISA detection of mycobacterial DNA in various clinical samples using IS990. The horizontal bar at an absorbance of 0.34 represents the minimum cutoff value for the designation of a positive result. Clinical samples categories: 1 – sputum; 2 – bronchoalveolar lavage; 3 – urine; 4 – pleural fluid; 5 – larynx swab; 6 – bronchial catheter; 7 – stomach lavage; 8 – cerebrospinal fluid; 9 – known negative controls (sputum); 10 – known negative controls (urine)

On the basis of these results the sensitivity of the test was found to be 96.5%, and the specificity was 95.3% for both IS*6110* and IS990 – targeted assays.

2.2. Epidemiological studies of *Mycobacterium tuberculosis* by restriction fragment length polymorphism (RFLP) analysis based on IS*6110*

The analysis of tuberculosis transmission and the tracing of the sources of infection require the ability to discriminate among *M. tuberculosis* strains. Several methods have been used for typing these strains. Until recently, all the methods relied on the study of phenotypic traits, such as serotype, biotype, bacteriophages sensitivity, or sensitivity to antimicrobial agents. These techniques have intrinsic limitations. For example, phenotypic expression may vary with culture conditions, and there are only a small number of phage types. To overcome these difficulties, genetic markers for the identification of strains within *M. tuberculosis* complex have been described. Insertion sequence IS*6110* has proved to be the most useful marker for epidemiological studies. Restriction fragment length polymorphism (RFLP) analysis based on IS*6110* has been useful for identifying transmission during outbreaks, for distinguishing between new infection and reactivation in patients who relapsed after treatment, and for the demonstration of cross-contamination of cultures in laboratories. Furthermore, an internationally accepted consensus for the standardized use of IS*6110* has been established (VAN SOOLINGEN et al. 1994).

In our epidemiological study, *M. tuberculosis* isolates from Polish TB patients between 1993 and 1996 were typed by the RFLP method, using a probe for IS*6110* and drug resistance profiles also were obtained (SAJDUDA et al. 1998). One hundred isolates of *M. tuberculosis* analyzed were collected at the Department of Microbiology at the Institute of Tuberculosis and Lung Diseases in Warsaw, the remaining 85 isolates were obtained in 1996 in the Hospital of Lung Diseases in Łódź. However, in some cases in which multiple isolates were from the same patient, each of the strains was identical by RFLP typing to the first isolate. Therefore, only 148 isolates from 148 patients were analyzed (Table 1). The species identification of the isolates was based on standard microbiologic tests. All the isolates were examined for their susceptibility to isoniazid, streptomycin, ethambutol and rifampin. The copy number of IS*6110* in each of the isolates was determined from the number of bands hybridizing to the probe, and ranged from 0 to 18. The DNA extracted from two isolates obtained from Polish patients did not hybridize with IS*6110* DNA. Also by PCR with the INS1-INS2 primers (specific to the IS*6110*) no amplifiable IS*6110* DNA was found in these strains. Hybridization patterns consistent with *M. tuberculosis* with the IS*1081* and DR-r probes (36 bp oligonucleotides flanking the IS*6110* element) were demonstrated for both of these strains. The majority, 126 of the 148 investigated isolates (85%), contained between 6 and 11 copies of IS*6110*. None of the strains carried only a single copy of the element. 91 different DNA fingerprint patterns were observed in the

96 isolates from Warsaw (the two isolates carrying no IS*6110* element were excluded). Of the 91 patterns, only four were shared by two or three isolates, whereas the remaining 87 isolates (90,6% of isolates) gave unique fingerprint patterns. The relatively low level of clustering (9,4%) likely reflects the wide geographic area from which these strains were derived. In contrast, among 50 strains isolated in Łódź, only 38 different fingerprint patterns were revealed. Of these 38 RFLP types, nine were shared by two strains, and one was identical for four strains. The remaining 28 patterns were unique.

Table 1: Polymorphisms of *M. tuberculosis* isolates from 146 TB patients in Poland during 1993–1996[a]

Year	Unique strains	No. of clusters	No. of strains in RFLP cluster	Total
Institute of Tuberculosis and Lung Diseases, Warsaw				
1993	5	1	2	7
1994	27	1	2	29
1995	55	1	2	60
		1	3	
Total	**87**	**4**	**2–3**	**96**
Hospital of Lung Diseases, Łódź				
1996	28	9	2	50
		1	4	
Total	**28**	**10**	**2–4**	**50**

[a] Excluding two isolates carrying no IS*6110* element.

IS*6110* DNA fingerprints of 42 drug-resistant isolates were compared with those of 99 drug- sensitive isolates. The result showed no noticeable difference in banding pattern or copy number of IS*6110* between drug-sensitive and drug-resistant isolates. Susceptible and resistant or even MDR (multidrug -resistant) strains were found in the same cluster.

Molecular techniques are used to track specific strains of pathogens and to determine more precisely the distribution of infectious diseases in populations, providing opportunities for more effective interventions. Currently, the most widely used genetic subtyping method for *M. tuberculosis* is restriction fragment length polymorphism (RFLP) analysis using the organism's repetitive DNA element IS*6110* (VAN SOOLINGEN et al. 1994). This method has been used to investigate outbreaks in health care settings and communities, to determine risk factors for active transmission of TB, to estimate the proportion of epidemiological links not identified by conventional contact tracing, to identify mis-diagnosis due to laboratory

cross-contamination and to quantify TB transmission between subpopulations. However, this RFLP typing method has several limitations. One major disadvantage is the time and labour required to perform the procedure. Moreover, strains with low (1–5) IS*6110* copy numbers, frequently found in some part of the world, or devoid of the element cannot be adequately analysed by this method. In this context, alternative polymerase chain reaction (PCR)-based techniques, including double-repetitive-element PCR (DRE-PCR), seem particularly promising. The DRE-PCR is a rapid subtyping method based on PCR amplification of *M. tuberculosis* DNA segments located between two repetitive elements: IS*6110* and the polymorphic GC-rich repetitive sequence, and it is recommended as a second-line test for analysis of *M. tuberculosis* isolates.

In this study we applied a standardized molecular strain typing method followed by DRE-PCR to characterize *M. tuberculosis* strains isolated from patients in Łódź area between 1996 and 2000 (DELA et al. in preparation). This analysis may provide a basis for estimating the relative importance, in this community, of recent transmission versus reactivation.

The isolates of *M. tuberculosis* analyzed in this study were collected in the Hospital of Lung Diseases in Łagiewniki, Łódź. This laboratory serves as a diagnostic service laboratory for the city and Łódź area. The isolates were randomly sampled from the beginning of 1996 to mid-2000. The species identification of the isolates was based on standard microbiological tests. All the isolates were examined for their susceptibility to ethambutol, isoniazid, streptomycin, and rifamipin with the BACTEC system. Only 4 strains (1.6%) were observed to be resistant to isoniazid (INH).

The 250 *M. tuberculosis* strains were analyzed by the RFLP technique. The copy number of IS*6110* in each of the strains ranged from 1 to 13. The majority, 212 of the 250 investigated strains (85%), contained between 6 and 11 copies of IS*6110*. 12% of the isolates were found to be low-copy-number strains (carrying from one to five copies of the IS*6110* element), including two strains harboring only a single copy of the insertion sequence.

In this study, 218 different IS*6110* DNA fingerprint patterns were observed in the 250 strains analyzed. Of the 218 patterns, 20 were shared by from two to five strains, whereas the remaining 198 patterns were observed only once each in this investigation. Fifty-two strains were included in 20 cluster patterns. One cluster occurred in 5 strains, two clusters in 4 strains, five clusters in 3 strains, and 12 clusters in 2 strains (Fig. 5). Despite the great diversity of the patterns among the isolates, a computer-assisted similarity analysis demonstrated a relatively high level of relatedness among a large number of different patterns. The majority of the patterns were included in several big clusters. The similarity among the individual non-identical patterns within each of these clusters ranged from 70 to 96% (Fig. 5).

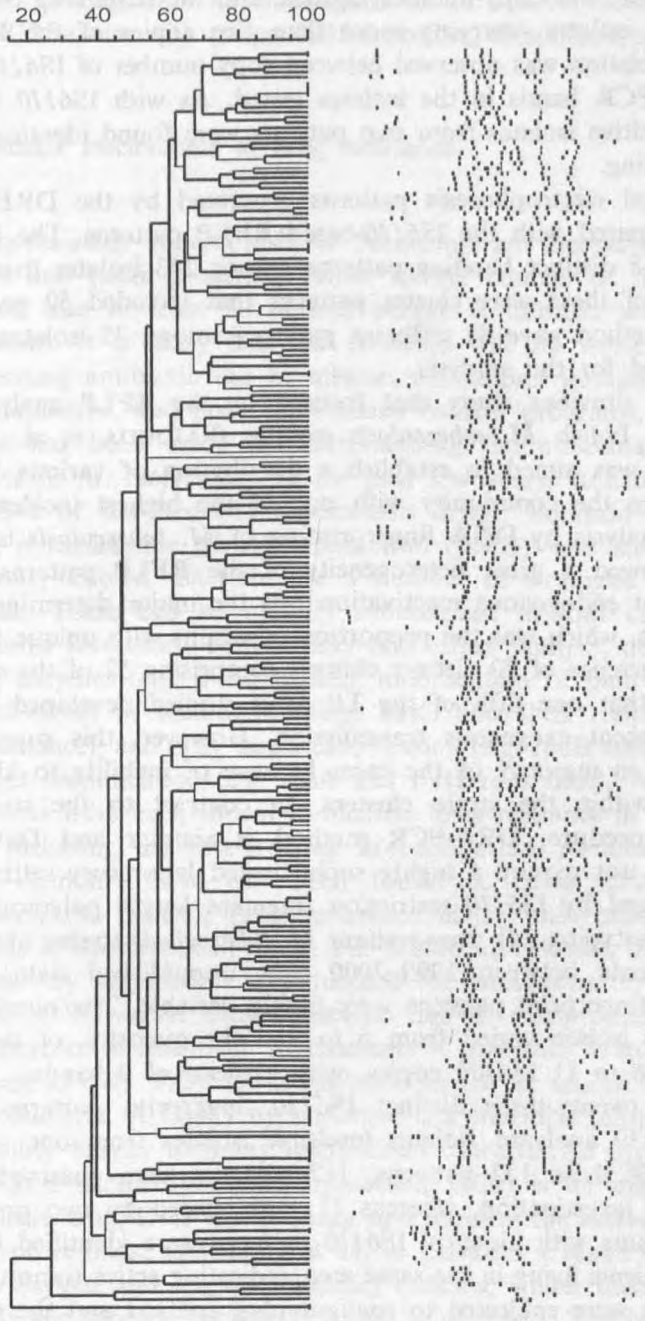

Fig. 5. Dendrogram based on computer-assisted comparison of IS6110 DNA fingerprints of 250 isolates of *M. tuberculosis*. Lane maps displaying the DNA fingerprints of the analyzed isolates are given at the right site of the dendrogram. The similarity among the patterns is shown above the dendrogram as a percentage

Seventy-five isolates from 73 patients were subject to DRE-PCR analysis. The 21 of these were low-copy-number isolates, and the remaining 54 were high-copy-number isolates (carrying more than five copies of the IS*6110* element). No correlation was observed between copy number of IS*6110* and number of DRE-PCR bands in the isolates tested. As with IS*6110* RFLP analysis, two repetitive isolates from two patients were found identical also by DRE-PCR typing.

The agarose gel electrophoresis patterns generated by the DRE-PCR method were compared with the IS*6110*-based RFLP patterns. The RFLP method yielded 218 distinct banding patterns among 263 isolates from 250 patients. Twenty of these were cluster patterns that included 52 patients. The DRE-PCR method gave 48 different patterns among 75 isolates from 73 patients selected for the analysis.

In contrast to previous study that focused on the RFLP analysis of randomly selected Polish *M. tuberculosis* isolates (SAJDUDA et al. 1998), the present study was aimed to establish a distribution of various RFLP types circulating in the community with one of the highest incidence of TB in Poland. Analysis by DNA fingerprinting of *M. tuberculosis* isolates in Łódź area showed a great heterogeneity of the RFLP patterns. The results suggest that endogenous reactivation was the major determinant of 79.2% of the cases, which was the proportion of strains with unique RFLP patterns. The occurrence of 20 distinct clusters comprising 52 of the strains (20.8%) suggests that one fifth of the TB cases studied developed active disease from a recent exogenous transmission. However, this suggestion cannot be proved in majority of the cases because of inability to identify patient contacts within the strain clusters. In contrast to the standard IS*6110* RFLP procedure, DRE-PCR method is simpler and faster to perform and may not require a highly sophisticated laboratory setting.

We also analyzed by IS*6110* restriction fragment length polymorphism (RFLP) and spoligotyping 141 drug-resitant *M. tuberculosis* strains obtained from Polish patients between 1999–2000 (our unpublished data). The generated IS*6110* fingerprint patterns were highly variable. The number of IS*6110* copies per isolate varied from 5 to 21. The majority, of the 139 strains contained 6 to 11 IS*6110* copies, with a mean of 9 bands.

One hundred twenty-three distinct IS*6110* fingerprint patterns were observed in the 139 analyzed isolates (multiple isolates from one patient were excluded). Of these 123 patterns, 112 patterns were observed only once each in this investigation, whereas 11 were shared by two or more isolates. Some strains with identical IS*6110* patterns were identified of the same family or patients living in the same area, indicating active transmission.

All the isolates were subjected to spoligotyping analysis and the results compared with those obtained by IS*6110*-associated RFLP. A total of 68

spoligotypes were identified in the 139 isolates. There were 9 spoligotypes identified among 27 isolates assigned to 11 IS*6110* clusters. In addition, among 112 isolates with unique IS*6110* profiles, 63 spoligotypes were identified.

3. Molecular mechanisms of drug resistance

Drug-resistant tuberculosis is becoming an increasing public health problem and poses a serious threat to the control of this disease. It is estimated that at least 50 million people is infected with drug-resistant tuberculosis. It is likely that this situation will get worse with the patter of increasing antibiotic use or misuse, the human immunodeficiency virus (HIV) pandemic, and poor tuberculosis control programs. A great deal of progress has been made in understanding the molecular basis of drug resistance in *M. tuberculosis* in the past few years. Resistance mechanisms to all five of the first-line tuberculosis drugs, isoniazid (INH), rifampin (RMP), pyrazinamide (PZA), ethambutol (EMB), and streptomycin (SM), are already known. Bacteria use a number of strategies to achieve drug resistance. These can be roughly summarized in four categories: barrier mechanisms (decreased permeability and efflux pumps), degrading or inactivating enzymes (e.g. β-lactamases), modification of pathways involved in drug activation or metabolism (e.g. katG and INH resistance, pacA and PZA resistance), and drug target modification (e.g. rpoB and RMP resistance) ora target amplification (e.g. inhA and INH resistance). While plasmids or transposons have been shown to mediate drug resisance in various bacterial species including the fast-growing *Mycobacterium fortuitum*, these mobile genetic elements have not heen found to cause drug resistance in *M. tuberculosis*. Instead, drug resistance in *M. tuberculosis* is caused by mutations in chromosomal genes. The multidrug-resistant (MDR) phenotype is caused by sequential accumulation of mutations in different genes involved in individual drug resistance due to inappropriate treatment or poor adherence to treatment. Mechanisms of resistance to tuberculosis-specific drugs such as INH, PZA and ethambutol (EMB) are unique to *M. tuberculosis* or mycobacteria. A highly hydrophobic cell envelope acting as an effective permeability barrier to many compounds characterizes mycobacteria.

Isoniazid (isonicotinic acid hydrazide, INH) is an important first-line tuberculosis drug. INH is a prodrug that requires the activation of bacterial catalase-proroxidase enzyme (Kat G) to generate a range of reactive (both reactive oxygen and reactive organic) radicals, which then attack multiple targets in the tubercle bacillus (ZHANG et al. 1992). The best known target is the cell wall synthesis pathway, where at least two enzymes, InhA (enoyl

acyl carrier protein (ACP) inductase), (BANERJEE et al. 1994) and KasA (beta-ketoacyl ACP synthase), (MDLULI et al. 1998), have been identified as targets for INH inhibition. It is important to note that INH has multiple effects on the tubercle bacillus, and it is not always easy to pinpoint which is the most essential target, whose inhibition will lead to cell death. During INH activation, the various reactive organic and reactive oxygen radicals produced could cause damage to various cellular targets, including DNA, carbohydrates, and lipids. In addition, INH has been proposed to affect NAD metabolism by incorporating into NAD through exchange with nicotinamide or by activating NAD glycohydrolase by removing its repressor, leading to NAD depletion (BEKIERKUNST et al. 1966).

Soon after INH was introduced in clinical treatment of tuberculosis, it was found that clinical isolates frequently lost catalase and peroxidase enzyme activities upon development of INH resistance. This observation led to the cloning of the catalase-peroxidase gene (*kat*G) and identification of *kat*G mutations (or deletion of the *kat*G) as the cause for INH resistance. Subsequent studies have shown that *kat*G point mutation is more frequent than deletions in INH-resistant strains. Mutation in KatG reduce its ability to activate the prodrug INH, thus leading to resistance. The *kat*G gene is situated in a highly variable region of the genome containing repeat DNA sequences, which may be the cause for the instability of this region and in turn may contribute to the frequency of *kat*G mutations in INH-resistant strains.

A reactive form of INH inhibits InhA by reacting with NAD(H) cofactor bound to the enzyme active site, forming a covalent adducts (isonicotinic acyl NADH) that is apt to bind with high affinity (ROZWARSKI et al. 1998). Resistance to INH can occur by mutations at the enzyme's active site that lower the enzyme's affinity for NADH without affecting the enzymatic activity or by increased expression of InhA (BASSO et al. 1998). In general mutations in *inh*A are associated with a low level of INH resistance. In contrast, mutations in *kat*G can cause either a low or high level of resistance, depending on the effect of mutations on the catalase-peroxidase enzyme activity required for INH activation. Mutations leading to complete loss of enzyme activity usually cause high level INH resistance. Mutations in InhA not only cause INH resistance but also confer resistance to the structurally related second-line antituberculosis drug – ethionamide. Mutations in kasA have so far been found in INH resistant as well as in INH susceptible strains (MDLULI et al. 1998). In most INH resistant isolates presenting *kas*A mutations, additional mutations have been identified in *kat*G or *inh*A. Thus the biological significance of *kas*A mutations needs careful definition.

Rifampin (RMP) is a broad-spectrum antimicrobial agent, which diffuses rapidly across the hydrophobic cell envelope. RMP interferes with synthesis of mRNA by binding to the bacterial DNA-dependent RNA polymerase. All bacteria (including mycobacteria) achieve resistance to RMP by a common strategy, mutation in a defined region of the RNA polymerase subunit β (RpoB). In the 81-bp core region of the RNA polymerase β subunit gene, mutations are observed with high frequency (HONORE, COLE 1993) (Fig. 6).

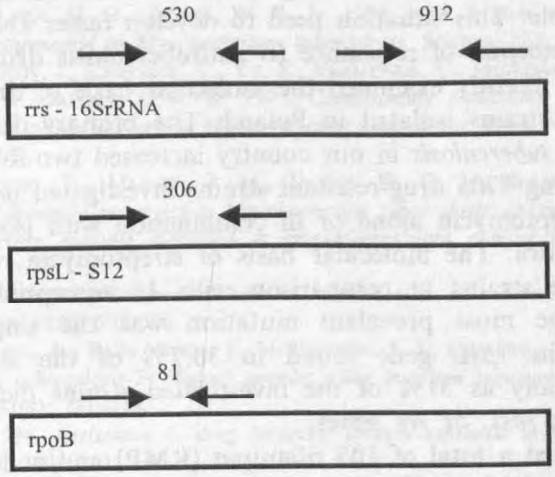

Fig. 6. DNA targets for rifampin and streptomycin. The most frequently mutated regions are indicated by arrows. See details in the text

Streptomycin (SM) inhibits initiation of mRNA translation and facilitates misreading of the genetic code and inefficient proofreading by the ribosome. The site of action is in the small 30S subunit of the ribosome, specifically at ribosomal protein S12 and 16S rRNA.

M. tuberculosis becomes resistant by mutating the target of SM in the ribosomes. The principal site of mutation is the *rps*L gene, encoding ribosomal protein S12 (FINKEN et al. 1993; SREEVATSAN et al. 1996). A second mechanism of resistance in *M. tuberculosis* is *rrs* (Fig. 6). While most bacteria have multiple copies of *rrs*, *M. tuberculosis* and other slow-growing mycobacteria are characterized by a single copy of *rrs* (BOTTGER 1994). Thus the loops of 16S rRNA that interacts with the S12 protein constitute an easily selected mutation site. Such mutations are clustered in the highly conserved 530 loop and on the adjacent 915 region (FINKEN et al. 1993). A third mechanism accounting for low-level resistance remains unidentified, but it has been suggested that it is derived from

Understanding the mechanisms of drug-resistance has practical application for rapid detection of drug-resistant tuberculosis by molecular methods. Genotypic analysis involves amplification by PCR of the genomic region conferring resistance, followed by postamplification analysis of mutations.

Recently, a world wide increase in the incidence of drug-resistant strains of M. tuberculosis was observed, especially multidrug-resistant (MDR-TB) isolates resistant to at least isoniazid and rifampin. The incidence of TB in Poland and also drug resistance is lower compared to the international data, but the importation of drug-resistant strains from former Soviet Union is possible. This situation need to develop faster and more accurate methods for detection of resistance to antituberculosis drugs (INH, RMP, SM). We have recently examined the molecular basis of drug resistance of *M. tuberculosis* strains isolated in Poland. The primary drug resistance of *Mycobacterium tuberculosis* in our country increased two-fold between 1997 and 2000. Among 3705 drug-resistant strains investigated in 2000, 169 were resistant to streptomycin alone or in combination with isoniazid, rifampin and/or ethambutol. The molecular basis of streptomycin resistance for 88 (52%) of these strains in comparison with 15 susceptible controls was determined. The most prevalent mutation was the single substitution Lys43Arg in the *rps*L gene found in 30.7% of the strains analyzed. However, as many as 51% of the investigated strains did not carry any mutation in the *rps*L or *rrs* genes.

We also tested a total of 105 rifamipin (RMP) and/or isoniazid (INH)-resistant strains of *Mycobacterium tuberculosis* for mutations associated with resistance to RMP and INH by sequence analysis. Three loci associated with drug-resistance were selected for characterization: *rpo*B (RMP), *kat*G and the regulatory region of *inh*A (INH). The most common point mutations were in codons 531 (41%), 516 (16%), and 526 (9%) of the *rpo*B gene. Mutations were not found in 2 (3%) of the isolates. In the case of resistance to INH, six different mutations in the *kat*G gene of 83 resistant strains were detected. Fifty-seven (69%) isolates exhibited nucleotide substitutions at codon 315. One strain harbored a mutation affecting codon 279 (Gly279Thr). Twelve of 26 INH-resistant strains with the wild-type codon 315 (14.5% of all strains tested) had mutation – 15C→T in the regulatory region of *inh*A. The detailed data of drug resistance analysis of MDR strains isolated in Poland will be published soon.

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