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SHORT COMMUNICATION

Epidemiological Analysis of *Mycobacterium tuberculosis* Strains Isolated from Patients of Small Communities Living in the South-East of Poland

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Abstract

The diversity of *Mycobacterium tuberculosis* clinical isolates, collected from a single hospital, was analyzed by ligation-mediated PCR techniques: FLiP and FLAP, and hybridization technique, IS*6110*-RFLP. The isolated strains were divided in terms of location (3 towns of Podkarpackie voivodeship differing in population size) and relationship (8 members of 4 families, each represented by 2 patients). Within each family identical DNA profiles, as well as drug resistance patterns were identified indicating a great chance of transmission of strains within the same family. Identical, or very similar patterns were also shared by strains isolated from unrelated patients living in a very small town (1 200 inhabitants) or hospitalized in the same place and time.

Key words: *Mycobacterium tuberculosis*, FLAP, FLiP, IS*6110*-RFLP, spoligotyping

Despite the fact that *Mycobacterium tuberculosis* (*Mtb*) is known as a causative agent of tuberculosis (TB) since 1882 (when it was first discovered), it remains a major threat to the public health all over the world. It is estimated that even one third of the world's population is infected with *Mtb* strains. According to World Health Organization, in 2012 around 8.6 million new tuberculosis cases and 1.3 million deaths caused by TB were registered worldwide (WHO Report, 2012). In Poland, in spite of observed decrement in the number of new cases, the incidence of TB is still considerably higher than in western European countries (19.6 vs 13.5 in 2012) (WHO Report, 2012). As reported in 2014, significant differences in morbidity between distinct regions of Poland were observed, varying from 10.6 (lubuskie) up to 30.2 (lubelskie) (Korzeniewska-Koseła, 2014); the incidence for the Podkarpackie voivodeship was 19.8 (Statistical Bulletin, Ministry of Health, 2013).

The frequency of *Mtb* transmission depends, among others, on population size. It is expected that transmission occurs more frequently in large populations in urban areas rather than in small towns or rural regions. Therefore, the prevalence of TB occurring among the inhabitants of the big cities is significantly higher than among the rural population (20.2 vs. 18.6 ratio) (Korzeniewska-Koseła, 2014). Additionally, the number of epidemiologically unrelated *Mtb* strains present within a large population is supposed to be much higher than in a small, local community.

The aim of this study was to analyze the diversity of *Mtb* strains isolated in 2012 and collected from 3 distinct towns of Podkarpackie region in Poland differing in population size (1 200, 30 000 and 60 000 inhabitants). Moreover, the epidemiological patterns of strains isolated within a few families of the same region were also identified.

The modern epidemiology of TB is based on molecular methods, which mainly focus on the diversity in number and localization of various repetitive DNA elements (Valcheva *et al*., 2008; Moström *et al*., 2002; Zozio *et al*., 2005). The most common techniques are spoligotyping, MIRU-VNTR typing and reference method IS6110-RFLP, which are indispensable for typing of large collections of strains (van Embden *et al*., 1993; Barnes and Cave, 2003; Crawford, 2003; Kremer *et al*., 2005; Supply *et al*., 2006; Covan *et al*., 2005). However, for an initial analysis of a limited number of strains or as a secondary methods, less laborious and cheaper

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methods could be applied, for instance ligation-mediated PCR methods, LM-PCR, which appeared to be highly discriminative (Masny and Płucienniczak, 2003; Krawczyk *et al*., 2006; Krawczyk *et al*., 2011, Zaczek *et al*., 2013a; 2013b; 2013c).

In the present study, *Mtb* clinical isolates were compared by means of two LM-PCR methods: FLiP (Reisig, 2005) and FLAP (Zaczek *et al*., 2014a; 2014b) and the obtained results were verified by the "gold" epidemiological standard IS*6110*-RFLP hybridization technique.

The 21 *Mtb* strains used in this analysis were isolated in 2012 from patients in the Podkarpackie voivodeship and hospitalized in the Independent Public Health Care Facility "Sanatorium" in Gorno. All strains were tested for susceptibility to isoniazid, rifampicin, pyrazinamide and ethambutol (Janowiec, 1988). The research material was sputum collected from patients of Polish nationality who were diagnosed with pulmonary tuberculosis. All patients, besides one, were newly detected cases. Detailed epidemiological data collected in course of community interviews, based on surveys, and from medical records are shown in the table (Table I). Genomic DNA was extracted and purified from all the isolates using the protocol proposed by van Embden (van Embden *et al*., 1993) and recommended for the standard IS*6110*-RFLP methods. The concentration of DNA was measured with NanoDrop ND-1000 spectrophotometer. Subsequently, isolates were characterized by IS*6110*-RFLP typing using internationally standardized protocol (van Embden *et al*., 1993). The FLAP method was performed as described previously (Zaczek *et al*., 2014a; 2014b) and the FLiP analysis was performed as originally described by Reisig (Reisig *et al*., 2005). The fingerprint patterns obtained by these three methods were compared visually with one another and strains were considered identical if their DNA profiles, obtained by all three methods, were the same.

The subject of the research were *Mtb* strains divided into two groups: first was comprised of the isolates taken from 13 unrelated patients living in 3 locations (A, B and C) in the Podkarpackie region, whereas the other consisted of 8 strains isolated from members of four families, each represented by two patients. From the location A, which counts over 60 000 inhabitants – 4 strains were analyzed, from the location B with 1 200 inhabitants – 2 strains were examined and from the location C with population 35 000 – 7 strains were analyzed, which represent 20%, 100% and 46% of all strains isolated in these cities in 2012, respectively.

The analysis of epidemiological patterns indicated that strains isolated in the location A represent identical banding patterns obtained by LM-PCR methods. However, banding profiles obtained with IS*6110*-RFLP proved that only three of these strains are identical, whereas the RFLP-IS*6110* pattern of the fourth strain

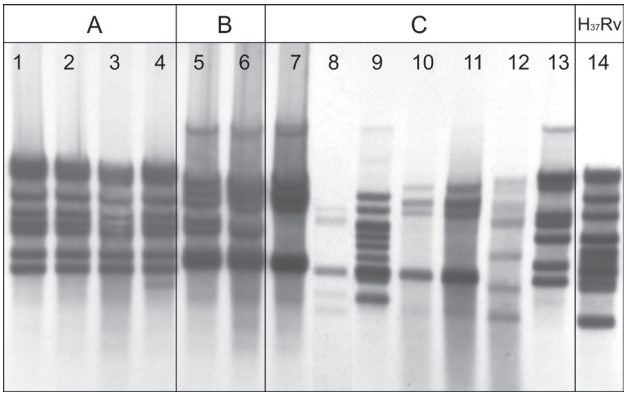

Fig. 1. DNA profiles obtained with IS*6110*-RFLP method. Line 1 to 4 – strains number 1, 2, 3, 4 from location A, line 5, 6 – strains number 9 and 10 from location B, line 7 to 13 – strains 13, 14, 15, 16, 17, 18, 19 from location C, line 14 – $H_{37}Rv$.

revealed one additional band suggesting a transposition of the mobile element (Fig. 1). Furthermore, this slightly different strain appeared to be sensitive to isoniazid (INH), while others were INH-resistant. In the case of 3 strains, due to their identical profiles obtained with three methods and the same drug resistance phenotype, it may be suspected that patients were in a close relationship or had accidental contact and there is a high probability of transmission of strains between them or a common source of infection.

Strains from location B were sensitive to all tested antimicobacterial drugs and identical in terms of DNA profiles obtained by the FLAP method. However, the DNA profiles obtained with FLiP and IS*6110*-RFLP methods (Fig. 1, lines 5 and 6) were slightly different (one additional band in strain number 9) what makes the direct transmission of these strains unlikely. On the other hand, the relationship between strains is clear and the common source of infection cannot be excluded. Among 7 strains analyzed from patients from the location C only two strains (Fig. 1, lines 10 and 11) showed the same molecular patterns obtained with three methods used, which indicates their great molecular affinity. However, some differences were observed in patterns of drug resistance. Strain 16 proved to be sensitive to the drugs used in the treatment of tuberculosis, while strain 17 showed resistance to streptomycin (SM) and INH. It is noteworthy that patients 16 and 17 live in the neighborhood, in the distance of approx. 500 m and on this basis, the transmission of strains between patients can be assumed as possible. Patient 17 could get infected by patient 16 or alternatively both patients could be infected from a common, unknown source. Other strains isolated from patients from this town showed different DNA profiles by means of all the methods used, confirming separate sources of infection and no transmission between patients. Out of 8 strains, isolated from patients who were members of 4 families,

Table I
Epidemiological data about strains based on surveys and from medical records.

Location/Family	Sample No	Sex/ Relationship	Age	AFB	Culture	Drug resistance				Drugs administered in treatment	Other Drugs	Comorbidities
						SM 4.0	INH 0.2	RFP 40.0	ETB 2.0			
A	1	F	31	+	+++	R	R	S	S	RFZ, PZA, EMB, SM, INH, Tarivid	Pyralgin, Gasec, Thiocodin, Hepatil, Allupol, Encorton	cholelithiasis, nephro-lithiasis of left kidney
	2	M	30	+	+	R	R	S	S	RMZ, PZA, EMB, Tarivid	Kalipoz, Nifuroksazyd, Insulatard, Novo-Rapid	diabetes type 1, alcohol dependence syndrome
	3	M	53	+++	+++	R	R	S	S	RMZ, PZA, EMB, SM	Pyralgin, Liv52, Acard, Kalipoz, Thiocodin, Contix, Allupol, Biodacyna	cardiorespiratory distress, alcoholism, nicotinism
	4	M	48	+	++	R	S	S	S	RMZ, PZA, EMB, INH	Hemofer, Liv52, Thiocodin, Exacyl, Pyralgina Cyclonamina	anemia
B	9	M	34	+	++	S	S	S	S	RMZ, PZA, EMB, SM	–	alcoholism, nicotinism
	10	M	22	+	$+^{10}$	S	S	S	S	RMZ, PZA, EMB	Cyclonamina, Pyralgin, Allupol	–
C	13	M	30	++	+++	S	S	S	S	RMP, PZA, SM	Cyclonamina, Exacyl, Thiocodin	fibro-infiltrative lesions of left lobe, alcoholism
	14	M	61	+	+++	S	S	S	S	RMP, PZA, SM	Pyralgin, Cyclonamina, 5%glukoza	fibro-infiltrative lesions of left lobe, cholelithiasis, drug-induced gastritis, nicotinism
	15	M	50	+	+++	S	R	S	S	RMF, PZA, SM, EMB, RMZ	Hemofer, Acard, Pyralgin	toxic liver damage, thrombosis of left leg, TB miliaris
	16	F	28	++	+++	S	S	S	S	RMZ,PZA, EMB	Contix, Nifuroxazyl, Allupol, Loperamid, PWE, Metronidazol	anemia, nicotinism
	17	F	50	+++	+++	R	R	S	S	SM, RMZ, PZA, EMB	PWE, Paracetamol, Biotrakson	secondary anemia, haemorrage into alvoli, nicotinism
	18	M	60	+	+++	S	S	S	S	SM, RMZ, PZA, EMB	Allupol, Promazin, Oxodil	manic-depressive disorder, nephrolithiasis, thyroid lumps, nicotinism
	19	M	52	(–)	$+^{12}$	S	S	S	S	SM, RMZ, PZA, EMB	Hemofer, Prol. Ac., Folicum, Allupol	nicotinism
D	7	M/ friend	53	+	++	S	S	S	S	PZA, EMB, Refalin, Tarivid	Gasec, Pyralgina, Allupol, Hepatil	alcohol-induced liver damage, chronic gastritis
	8	M/ brother	52	+	+++	S	S	S	S	RMZ, PZA, EMB, SM	Pyralgin, Liv52, Acard, Kalipoz, Thiocodin, Contix, Allupol, Biodacyna	cardiorespiratory distress, alcoholism, nicotinism
E	32	M/son	61	+++	+++	S	S	S	S	RMZ, PZA, EMB, SM	Allupol, Amlopin Alermed, Contix, Loperamid	hypertension, alcoholism, nicotinism
	33	M/ father	40	+	+++	S	S	S	S	RMZ, PZA, EMB, SM	Ketokonazol, Flukonazol, Paracetamol	mitral regurge, pulmonary embolism, alcoholism, nicotinism
F	6	M/ brother	61	+	+	S	S	S	S	RMZ, PZA, EMB, INH	Zafiron, Acenol, Hemofer	chronic obturative pulmo-nary disease, secondary anemia, pneumonia, alcoholism, nicotinism
	23	M/ brother	51	+	+	S	S	S	S	RMZ, PZA, EMB, SM	Hemofer, Folicum, Flegamina	fibro-infiltrative TB of left lobe
G	30	K/ mother	54	+	+	S	S	S	S	RMZ, PZA, EMB, SM	Kalipoz, Poltram, Bisocard	infiltrative TB of lungs, alcoholism, nicotinism
	31	M/son	31	+	+	S	S	S	S	RMZ, PZA, EMB	Contix, Estazdom, Allupol	secondary anemia, alcoholism, nicotinism

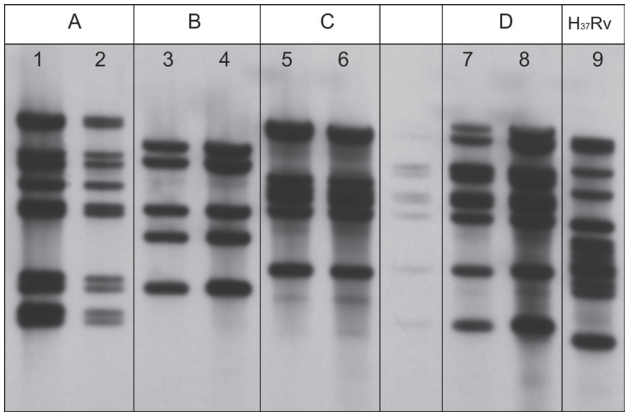

Fig. 2. DNA profiles obtained with IS*6110*-RFLP method. Line 1 and 2 – strains number 7 and 8 from family D, line 3, 4 – strains number 32 and 33 from family E, line 5, 6 – strains 6 and 23 from family F, line 7 and 8 – strains 30 and 31 from family D, line 9 – $H_{37}Rv$.

4 epidemiological groups corresponding to 4 analyzed families were distinguished. Within each family identical DNA profiles, as well as drug resistance patterns were identified (Fig. 2, Table I). This indicates a great chance of transmission of strains within the same family. A similar situation was observed in a small town, where the contact with infected persons is very likely.

Properly conducted tuberculosis supervision includes, apart from identification of the source of infection, tracking the ways of transmission of strains in the environment. It is known that the best way of tuberculosis prevention and its surveillance are primarily: detection of sputum positive Tuberculosis patient, integration of antituberculous treatment and examination of all the people around the patient in order to detect or exclude the contagion (Kozińska *et al*., 2011). Molecular methods used in the epidemiology of tuberculosis allow for prompt recognitions of specific strains and monitor the transmission of the disease. However, those methods should be supported by socio-demographic data that are substantial for identification of epidemiological groups in which mycobacteria could be transmitted. These include *i.a.*: a degree of kinship of people diagnosed with mycobacteria, the social status of the subjects, comorbidities, place of residence, *etc.* The transmission of strains is greater when the contact is more direct, primarily in close proximity to the patient (family, closed groups, small communities, prisons, health centers). However, even identical molecular formulas of strains may not constitute sufficient evidence that the source of infection is a person from the immediate surroundings. In this report, the molecular part of epidemiological investigations was based on 3 different methods. It should be noted that only data obtained by means of several methods of strains differentiation accompanied by detailed social history may show the actual transmission of individual strains. On the other hand, if different molecular patterns are obtained with the aforementioned methods, it allows for unambiguous interpretation of the results and the exclusion of transmission of strains between patients from whom they were isolated.

Analysis of the strains was used to assess the degree of TB spreading among subjects and point to the likelihood of strain transmission among patients. In all studied cases of families a high probability of transmission was observed. Also, the analysis of strains from patients residing in a small town (1 200 inhabitants) indicated a direct transmission between patients or a common source. On the other hand, out of 7 strains from location C only 2 were epidemiologically linked. In numerous community contacts a potential source of infection can be random and more unrelated *Mtb* strains might be present. The tested strains of location A accounted for 20% of all registered cases of tuberculosis in this town in 2012, and this was probably not a very representative group, and patients could come from a single place, *e.g.* from one surgery. More general conclusions about the level of transmission of *Mtb* strains require performing a molecular analysis of strains isolated over a number of years.

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