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Phenotypic characterization of pyrazinamide-resistant *Mycobacterium tuberculosis* isolated in Poland

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Abstract

Introduction: Pyrazinamide (PZA) is an important first-line anti-tuberculous drug, which is applied together with INH, RMP, EMB, and SM. This drug plays a unique role in the first phase of TB therapy because it is active within macrophages and kills tubercle bacilli. Testing the resistibility of *Mycobacterium tuberculosis* to PZA is technically difficult because PZA is active only at acid pHs. Therefore, routine drug resistibility testing of *M. tuberculosis* for PZA is not performed in many laboratories. The objective of our study was to estimate the resistibility for PZA among *M. tuberculosis* isolates from Polish patients in the years 2000–2008.

Material and methods: We analyzed *M. tuberculosis* strains with different resistibility to first-line anti-tuberculous drugs. The strains were isolated from 1909 patients with tuberculosis. The strains were examined for PZA resistibility by the radiometric Bactec 460-TB method. The PZA-resistant strains were examined for the following MIC PZA for drug concentrations: 100, 300, 600, 900 µg/mL.

Results: PZA resistance among *M. tuberculosis* strains was found in 6.7% of untreated patients and in 22.2% of previously treated patients ($p < 0.001$). In both groups, resistance to PZA correlated with drug resistance for INH+RMP+SM+EMB — in 32.7% of untreated patients and in 34.5% previously treated patients ($p < 0.8$). PZA-monoresistant strains were observed in 20.8% of untreated patient groups. Among the resistant strains: in 3.4% MIC for PZA was > 100 µg/mL, in 11.6% ≥ 300 µg/mL, in 8.9% ≥ 600 µg/mL, and in 76% ≥ 900 µg/mL.

Conclusions: Among *M. tuberculosis* strains, PZA resistance was found in 6.7% of untreated patients and in 22.2% of previously treated patients. Among the PZA-resistant strains, very high MIC values for PZA (≥ 900 µg/mL) were revealed for 76% *M. tuberculosis* strains.

Key words: tuberculosis, *Mycobacterium tuberculosis*, PZA-resistance

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Introduction

The anti-tuberculous properties of pyrazinamide (PZA) were identified over 50 years ago, and since that time it has been one of the most important drugs used for the treatment of tuberculosis, in addition to rifampicin (RMP), isoniazid (INH), streptomycin (SM), and ethambutol (EMB). Although PZA plays such an important role in the treatment of tuberculosis, the World Health Organisation (WHO) has not issued a recommendation to collect data on the prevalence of

PZA resistance among *Mycobacterium tuberculosis* strains or to perform susceptibility testing for this drug. This mainly results from the difficulty of the PZA susceptibility test. It is therefore unknown how prevalent PZA resistance is worldwide [1].

Pyrazinamide is a drug which demonstrates its anti-tuberculous activity in acidic conditions (pH 5.0–6.0), which is why it is very potent inside macrophages and necrotic masses. Structurally, PZA is an analogue of nicotinamide. Like isoniazid, PZA is a prodrug and is converted to the active form by the

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enzyme pyrazinamidase produced by mycobacteria. The enzyme deaminates the drug to pyrazinoic acid (POA). Neither the biochemical principles of these reactions nor the mechanism of action on intracellular structures are unclear, although some effect of the drug on fatty acid synthesis has been suggested [2–5]. It has been observed that at acidic pH the absorption and accumulation of POA in mycobacterial cells increases, which blocks the active and passive transport in the cell [6]. Assuming that the effects of POA are intended to impair fatty acid synthesis [7], POA, a weak acid, could potentially kill mycobacteria by altering cell membrane potential [8].

Susceptibility to PZA correlates with the activity of amidase, which is decreased or absent in most resistant strains, as is the case with the naturally PZA-resistant strains of *M. bovis* and *M. bovis* BCG. This feature is particularly useful in differentiating between *M. tuberculosis* and *M. bovis*.

Tuberculosis caused by *M. bovis*, which is mainly a veterinary problem, may also be transmitted between humans [9]. Tuberculosis caused by *M. bovis* is detected in humans with HIV infection [10, 11]. In many developing countries, such as Latin America, tuberculosis caused by *M. bovis* accounts for about 2% of new cases of pulmonary tuberculosis and 8% of extrapulmonary cases [12]. In regions where tuberculosis in humans and animals co-exists and causes endemics, precise differentiation between *M. bovis* and *M. tuberculosis* is essential for monitoring the spread and human transmission of bovine tuberculosis. The most important reason why it is necessary to differentiate between the two species is the different management of tuberculosis caused by *M. bovis*. *M. bovis* is naturally resistant to PZA, which is why the drug must be excluded from the treatment regimen [13].

The aim of the study was to retrospectively analyse the prevalence of PZA resistance in the collection of *M. tuberculosis* strains and to determine PZA MIC values.

Material and methods

We analysed *M. tuberculosis* strains isolated and shipped to the Department of Microbiology between the years 2000 and 2008 from 1909 patients originating from various regions of Poland. Most patients originated from the Mazovian, Kuyavian-Pomeranian, and Malopolskie provinces (17.8%, 16.3%, and 9.5%, respectively).

Among the 1909 patients there were 1289 (67.5%) men and 505 (26.5%) women aged 6–95 years. There were 1517 (79.5%) newly diagnosed patients and 392 (20.5%) previously treated patients.

All the strains were cultured and drug resistance tested using standard methods. The species were determined using the niacin test and spoligotyping.

Spoligotyping is a genetic method based on the polymorphism of the direct repeat (DR) region characteristic of *M. tuberculosis* complex. The number of DR sequence copies present in the genome of mycobacteria is characteristic of a given strain.

Based on the obtained molecular pattern, the method allows identification of strains within the *Mycobacterium tuberculosis* complex, namely: *M. tuberculosis*, *M. africanum*, *M. canetti*, *M. microti*, *M. bovis*, *M. bovis* BCG, *M. caprae*, and *M. pinnipedii*. The principle of spoligotyping and its use have been described elsewhere [14, 15].

Resistance of the strains to the four principal anti-tuberculous drugs was determined using the classical method in the Loewenstein-Jensen medium and on the BACTEC 460TB system.

The phenotype of PZA resistance of the strains was determined radiometrically using the BACTEC 460TB system, in the liquid Middlebrook medium 7H12 at a pH of 5.9–6.0 and at PZA threshold concentration of 100 µg/mL. We used the same system to determine PZA MIC values for the following drug concentrations: 100, 300, 600, and 900 µg/mL.

We used the *t*-Student test to assess the significance of the differences.

Results

In the group of 1909 patients from whom *M. tuberculosis* was isolated there were 188 (9.8%) patients with strains resistant to PZA. Identification by spoligotyping and the niacin test showed that all the PZA resistant strains were *M. tuberculosis*. These strains became the starting point for further analyses.

In the group of 1517 of patients newly diagnosed to have been infected with *M. tuberculosis* there were 101 (6.7%) patients infected with strains resistant to PZA. Resistance to this drug in the group of previously treated patients (392), eliminating *M. tuberculosis*, was observed in 87 cases (22.2%). The prevalence of PZA resistance in both groups differed significantly ($p < 0.001$) (Fig. 1).

We also analysed resistance to other principal anti-tuberculous drugs with which PZA resistance was associated.

Analysis of resistance to PZA in the group of newly diagnosed patients

In the group of 101 newly diagnosed patients, 33 patients (32.7%) showed resistance to PZA accompanied by resistance to SM + INH + RMP +

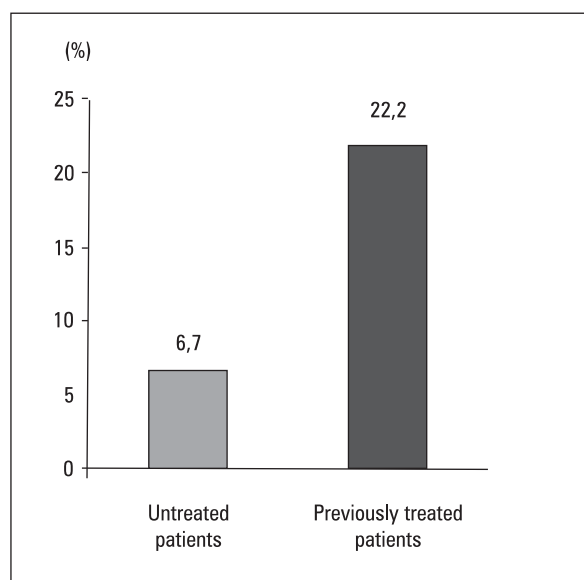


Figure 1. PZA-resistance among *Mycobacterium tuberculosis* strains isolated from tuberculosis patients

+ EMB, 12 patients (11.9%) showed resistance to PZA accompanied by resistance to SM + INH + RMP and INH + RMP, and 3 patients (3%) showed resistance to PZA accompanied by resistance to INH + RMP + EMB. In 20 patients (19.8%), resistance to PZA accompanied non-MDR resistance and consisted of resistance to SM or SM + INH or SM + INH + EMB. In the group of newly diagnosed patients, there were 21 patients (20.8%) whose mycobacteria were resistant to PZA only.

Analysis of resistance to PZA in the group of previously treated patients

In the group of 87 previously treated patients infected with *M. tuberculosis* resistant to PZA, the following patterns of resistance predominated: SM + INH + RMP + EMB (30 patients [34.5%]), SM +

+ INH + RMP (25 patients [28.7%]), INH + RMP (14 patients [16.1%]), and INH + RMP + EMB (7 patients [8%]). The lowest percentage was found among strains resistant to the 4 principal drugs and resistant to PZA (3 patients [3.4%]) (Table 1).

When we compared the resistance patterns of PZA-resistant *M. tuberculosis* strains isolated from newly diagnosed patients and previously treated patients we concluded that in both groups resistance to four drugs predominated (INH + RMP + SM + EMB) (32.7% and 34.5%, respectively, $p < 0.8$). Strains resistant to PZA only predominated in the group of newly detected patients (20.8% v. 3.4%). The prevalence of PZA resistance significantly differed between the groups ($p < 0.001$).

The fewest newly diagnosed patients and previously treated patients eliminated *M. tuberculosis* resistant to INH + RMP + EMB + PZA (3.0% v. 8.0, $p < 0.2$) (Fig. 2).

Analysis of PZA MIC values for *M. tuberculosis* strains

PZA MIC values were determined for 146 strains of *M. tuberculosis* resistant to this drug. MIC values were $> 100 \mu\text{g/mL}$ for 5 strains (3.4%), $\geq 300 \mu\text{g/mL}$ for 17 strains (11.6%), and $\geq 600 \mu\text{g/mL}$ for 13 strains (8.9%). The highest MIC values ($\geq 900 \mu\text{g/mL}$) were found in 111 strains (76%) (Fig. 3).

Discussion

Determination of resistance of *M. tuberculosis* strains to PZA is one of the most difficult microbiological tests [16]. Conventional drug resistance testing for *M. tuberculosis* may be completed within 7–28 days, depending on the culture system used [17]. For the majority of drugs the tests are standardised and results reproducible. In the case

Table 1. Frequency of PZA-resistance among *Mycobacterium tuberculosis* strains with different susceptibility on first-line antituberculous drugs

Patter of resistance	No of strains resistant to PZA		t-Student test
	Untreated patients	Previously treated patients	
SIRE	33 (32.7%)	30 (34.5%)	$p < 0.8$
SIR	12 (11.9%)	25 (28.7%)	$p < 0.01$
IRE	3 (3.0%)	7 (8.0%)	$p < 0.2$
IR	12 (11.9%)	14 (16.1%)	$p > 0.4$
Sensitive	21 (20.8%)	3 (3.4%)	$p < 0.001$
Others	20 (19.8%)	8 (9.2%)	$p < 0.05$
Total	101 (100%)	87 (100%)	$p < 0.2$

S — streptomycin; I — izoniazid; R — rifampicin; E — ethambutol

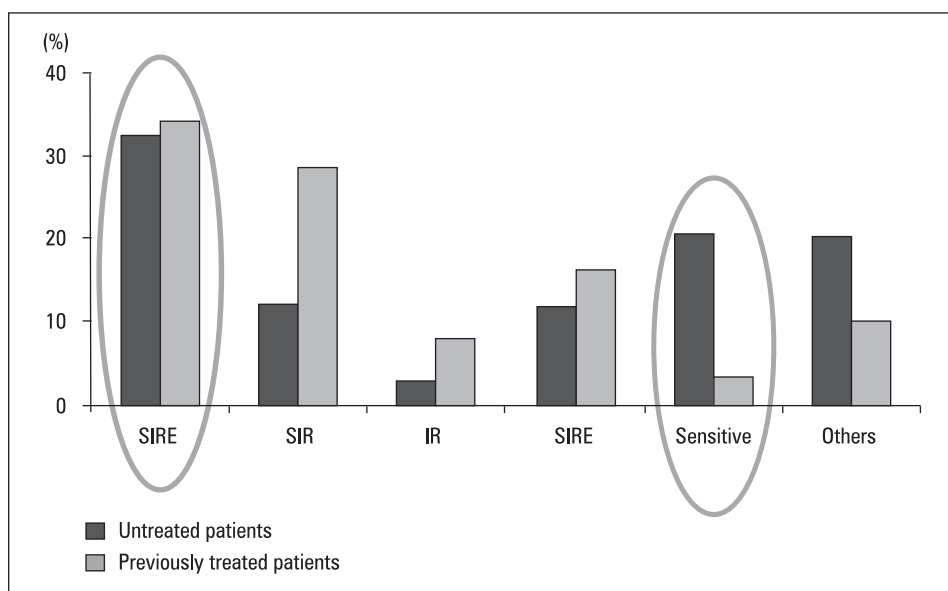


Figure 2. PZA-resistance among *Mycobacterium tuberculosis* strains in relation to different susceptibility to first-line antituberculous drugs isolated from untreated and previously treated patients

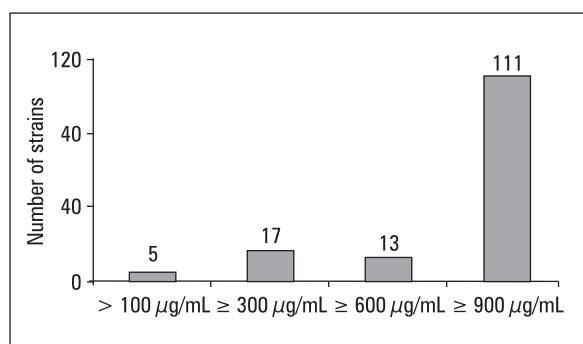


Figure 3. MIC value for PZA [μg/mL] for 146 *Mycobacterium tuberculosis* strains examined by the Bactec 460 Tb method

of PZA, the drug's activity correlates with the pH of the cultures, as a result of which the drug is most active at pH 5.5, less active at pH 6, and completely inactive at neutral pH [18].

Problems with keeping culture conditions necessary for the preservation of anti-tuberculous activity of PZA (pH 5.5 ≤ 6.0) along with the preservation of optimal conditions for mycobacterial growth (pH of about 7.0) are well known. It is estimated that resistance testing cannot be performed for at least 10% of strains from clinical isolates because they fail to grow at such low pHs of the cultures [19]. Problems with the correct performance of PZA resistance testing can also result from using an excessive inoculum, which promotes aggregation of the mycobacterial cells leading to elevated pH of the cultures in which the test is being carried out. A similar effect may be visible in liquid media containing serum or

albumin complex, in which POA may be bound with proteins and therefore inactivated. Furthermore, susceptibility to PZA depends on the growth phase of the mycobacteria. Ageing 3-month-old cultures of the H37Ra strain were more susceptible to PZA than young 4-day-old cultures in the logarithmic growth phase. A great deal of information about the mechanisms of mycobacterial resistance to PZA has been provided by efflux pump studies [12, 20–22].

It is not only a problem to maintain a low pH in the cultures but also to establish the value of the critical concentration that would enable phenotypic determination of drug resistance. The implementation of the BACTEC 460TB system has enabled standardisation of the method although the PZA resistance test continues to be one of the most difficult ones [19, 23].

Despite years of studies, there is no consensus as to the criterion of mycobacterial resistance to PZA *in vitro*. The drug's critical concentration is 25 μg/mL in the Middlebrook Cohn 7H10 agar medium and 100 μg/mL in the BACTEC system [12, 24]. Now that it has been discovered that such mutations as *pncA* gene mutations are responsible for resistance to PZA, it has become easier to observe correlations between MIC values and the presence of mutations and to draw conclusions as to whether the critical MIC values should be changed in the various microbiological methods.

In our study, in the group of 1909 patients with tuberculosis, 188 patients eliminated mycobacteria resistant to PZA; therefore, their growth was not inhibited at the threshold concentration of 100 μg/

mL, as confirmed by evaluation of MIC values for these strains. Among the analysed strains, titres of resistance to PZA were high, with about 80% showing the highest MIC value of $\geq 900 \mu\text{g/mL}$ and only 3.4% (5 strains) showing MIC values slightly above than the critical value (PZA MIC $> 100 \mu\text{g/mL}$).

Verification of the reliability of drug resistance tests conducted in California showed that among 1916 strains of *M. tuberculosis* defined as resistant to PZA by the laboratories 14 (0.7%) were actually susceptible to this drug [25]. The incorrectly evaluated strains included 2 strains belonging to another species, namely *M. bovis* and *M. bovis* BCG, which — as is widely known — are naturally resistant to PZA. In addition to the incorrectly evaluated strains, three strains of *M. tuberculosis* resistant to PZA only were found [26]. Monoresistance to PZA is not always a marker of *M. bovis* as it may also be observed in *M. tuberculosis*. In view of this, resistance to PZA can no longer be considered a certain test for identification of *M. bovis* [22, 27].

Species identification of the genetically related *M. tuberculosis* and *M. bovis* is necessary due to their differing significance for the epidemiology and management of tuberculosis in humans [10, 26, 28]. In our study, all the analysed strains were identified, using biochemical and molecular methods, as *M. tuberculosis*.

The analysis of our material showed that strains resistant to PZA isolated from 24 patients were susceptible to the four principal anti-tuberculous drugs. There were 21 (20.8%) such strains in the group of newly diagnosed patients and only 3 (3.4%) in the group of previously treated patients. The high percentage of newly diagnosed patients eliminating PZA monoresistant mycobacteria indicates that these cases result from transmission from the environment and not from incorrect treatment.

PZA monoresistance has also been reported in a cohort study of *M. tuberculosis* strains isolated from patients in Canada [29], which demonstrated that the strains isolated from 21 patients (20 patients with PZA monoresistance and 1 patient with MDR) not only contained the same mutation of the *pncA* gene, but were also characterised by the same RFLP pattern, which confirmed transmission of the same strains among these patients. The next step in the analysis of strains resistant to PZA isolated from Polish patients will involve examining genetic relationships between these strains [30].

It is surprising that resistance to PZA among the strains isolated in Poland is relatively high (6.7% in newly diagnosed patients and 22.2% in previously treated patients) compared to resistance to other first-line drugs. When we analysed the

prevalence rate of resistance to PZA among MDR strains in both study groups we found it to be very high (about 70%). How could one explain this very high rate of resistance to PZA in Poland, where resistance of *M. tuberculosis* to other drugs is not as frequent as in other Eastern European countries?

One should remember that the prevalence of the natural mutations of PZA resistant mycobacteria is higher than that for the other drugs and equals $1/10^4$. In extensive tuberculous lesions, in which the population of mycobacteria is numerous (e.g. 10^{10} – 10^{14}), selection of the naturally resistant mutants happens very easily and rapidly [31, 32].

Studies in animal models of tuberculosis have demonstrated that this valuable anti-tuberculous drug could only be used for 2–3 months because of the easy and rapid emergence of drug resistance [32]. Extending the duration of treatment potentiates the phenomenon of drug resistance.

Resistance to PZA may also result from using the drug alone in the treatment of tuberculosis. As is commonly known, treatment of tuberculosis requires a combination of 3–4 drugs, particularly in extensive disease, as is the case in the initial phase of the treatment. Our study and observations made by pulmonologists suggest that PZA is given for much longer periods of time in Poland, e.g. for 12 months, even in monotherapy [33].

As the first PZA resistance studies in Poland [31] showed its high prevalence. Zwolska [34] proposed that the PZA resistance test, which can be performed at several laboratories in Poland, should be carried out routinely as part of the basic susceptibility testing.

Conclusions

1. Resistance to PZA was found in *M. tuberculosis* strains isolated from 6.7% (101) of newly diagnosed patients and from 22.2% (87) of previously treated patients. No cases caused by *M. bovis* or *M. bovis* BCG were observed.
2. Resistance to PZA often accompanied resistance to the four principal anti-tuberculous drugs (SM + INH + RMP + EMB) in the group of newly diagnosed patients (32.7%) and the group of previously treated patients (34.5%).
3. Resistance to PZA was also observed in strains susceptible to the four principal anti-tuberculous drugs. The number of such cases among the newly diagnosed patients was over 6-times higher than that among the previously treated patients, which points to the infection with strains resistant to PZA and requires further molecular studies of their transmission.

4. In view of the above, it should be emphasised that the PZA resistance test cannot continue to be considered a test that differentiates between *M. bovis* and *M. tuberculosis*.
5. Among the strains resistant to PZA, PZA MIC was $\geq 900 \mu\text{g/mL}$ in 80% of the strains.
6. The PZA resistance test should be included in the basic susceptibility testing in patients newly diagnosed with tuberculosis.

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