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Use of GenoType MTBDR plus assay for the detection of mycobacteria molecular rifampicin and isoniazid resistance

Zastosowanie testu GenoType MTBDR plus do wykrywania molekularnych mechanizmów oporności na ryfampicynę i izoniazyd wśród prątków gruźlicy

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Mycobacterium tuberculosis, rifampicin, isoniazid, multidrug resistance

Słowa kluczowe

Mycobacterium tuberculosis, ryfampicyna, izoniazyd, wielolekooporność

S u m m a r y

Introduction. The main causes of drug-resistant TB is the use of improper treatment regimens, and incorrect diagnosis of patients. The rapid identification of drug resistance strain is an important challenge to ensure a rapid and adequate therapy of tuberculosis and to limit the dissemination of multidrug resistant strains. Isoniazid (INH) and rifampicin (RMP) are the main antituberculosis drugs, used in the treatment regimen recommended by the WHO. The occurrence of resistance to these two basic medicines are considered to be one of several causes of treatment failure in patients with tuberculosis.

Aim. The aim of this study was to compare the results of resistance to isoniazid and rifampicin obtained with the molecular and the phenotypic method.

Material and methods. The MTBDR plus assay was performed on 60 strains *Mycobacterium tuberculosis* complex, and the results were compared with the results of conventional drug susceptibility testing (Bactec MGIT 960).

Results. In the analyzed group of 60 strains of mycobacterium tuberculosis, mutations in the *rpoB* gene were detected in 40 of 42 strains of *M. tuberculosis* phenotypically resistant to RMP (92.9%). In the group of 51 *M. tuberculosis* strains phenotypically resistant to INH, mutations in the *katG* gene and *inhA* gene were found in 46 (90.2%). The sensitivity, specificity and positive and negative predictive values of the MTBDR assay were respectively 82.9, 94.7, 97.1 and 72% for multidrug-resistant TB (MDR-TB).

Conclusions. The GenoType MTBDR plus assay is a molecular test which detecting the most common mutations in strains resistant to RMP and INH. However the test does not detect all mutations associated with resistance to isoniazid and rifampicin, therefore, the results of molecular must be confirmed by phenotype.

S t r e s z c z e n i e

Wstęp. Jedną z przyczyn występowania gruźlicy lekoopornej jest długotrwała diagnostyka chorych i trudności w akceptacji schematów leczenia przez chorych. Szybka identyfikacja lekoopornych szczepów *M. tuberculosis* complex pozwala na włączenie odpowiedniego schematu leczenia, co w konsekwencji zapobiega transmisji szczepów. Izoniazyd (INH) i ryfampicyna (RMP) są głównymi lekami przeciwprątkowymi, stosowanymi w schemacie leczenia rekomendowanym przez WHO. Wystąpienie oporności na te dwa podstawowe leki uznaje się za jedną z kilku przyczyn niepowodzenia leczenia chorych na gruźlicę.

Cel pracy. Celem pracy było porównanie wyników testu lekooporności szczepów *M. tuberculosis* complex na izoniazyd i ryfampicynę uzyskanych metodą fenotypową i molekularną.

Materiał i metody. Analizie poddano 60 szczepów *Mycobacterium tuberculosis* complex, u których wykonano test lekooporności na RMP i INH metodą molekularną GenoType MTBDR plus oraz metodą fenotypową z zastosowaniem systemu Bactec MGIT 960.

Wyniki. W analizowanej puli 60 szczepów prątków gruźlicy mutacje w genie *rpoB* warunkujące oporność na RMP wykryto u 40 z 42 szczepów *M. tuberculosis* fenotypowo opornych na RMP (92,9%). W puli 51 szczepów fenotypowo opornych na INH mutacje w genach *katG* i *inhA* warunkujące oporność stwierdzono u 46 szczepów (90,2%). Czułość, specyficzność oraz dodatnia i ujemna wartość predykcyjna testu GenoType MTBDR plus dla szczepów o oporności MDR (MDR-TB) wynosiły odpowiednio: 82,9, 94,7, 97,1 i 72%.

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Wnioski. Test GenoType MTBDR plus jest testem molekularnym wykrywającym najczęściej występujące mutacje w genach odpowiedzialnych za oporność na RMP i INH. Ponieważ jednak nie wykrywa on wszystkich mutacji związanych z opornością na oba leki, wyniki badań molekularnych muszą zostać potwierdzone metodą fenotypową.

INTRODUCTION

The most important reasons for the worsening situation of the epidemiology of tuberculosis in the world include: poor disease control programs and their inadequate implementation, ignoring the problem of tuberculosis in developed countries, the lack of resources for treatment of patients in developing countries, the spread of HIV. The phenomenon of drug resistance of mycobacteria has been recognized by WHO experts as one of the major reasons for the severity of tuberculosis in the modern world.

The World Health Organization reported, in 2013, 480 000 new cases of MDR TB (MDR-TB) resistant to at least isoniazid (INH) and rifampicin (RMP). It is estimated that, worldwide, TB patients excreting MDR (multidrug resistant) tubercle bacilli represent 3.5% of the new patients and 20.5% of the previously treated group. The incidence of drug resistance is a primary indicator of the effectiveness of epidemiological surveillance and treatment of tuberculosis in societies and acquired drug resistance shows the correctness of the treatment of various groups of patients. The largest number of cases of MDR-TB is recorded in Eastern Europe and Central Asia. There, patients with MDR-TB represent over 20% of the new patients and more than 50% of the previously treated patients (1). I**n Poland, according to the Central Register of Tuberculosis, in 2013, 40 patients with MDR tuberculosis were registered, representing 0.9% of all of the registered cases of tuberculosis (2). Tuberculosis resistant to drugs, and particularly MDR-Tb and XDR-Tb, is a serious problem affecting the health of humans and implementation of programs to combat tuberculosis. MDR-TB and XDR-TB are highly fatal diseases, with a mortality rate of about 60%.**

Information on resistance patterns of *Mycobacterium tuberculosis* strains isolated from patients is an important element of supervision of tuberculosis. Research and analysis of the frequency of drug-resistant TB are helpful in detecting and monitoring the spread of MDR and XDR strains and illustrate the effectiveness of surveillance of tuberculosis in the given country. At the moment, in laboratories around the world, a major challenge is rapid identification of drug-resistant strains of *Mycobacterium tuberculosis* complex. Early detection and diagnosis of patients with this form of tuberculosis enable appropriate treatment regimen which reduces the amount of mycobacteria and patient infectivity and prevents the transmission of drug-resistant strains in the human environment.

The tests used for the determination of drug resistant *M. tuberculosis* completely differ from tests performed for other bacteria. This is mainly due to the physiological and biochemical differences of mycobacteria. Development of rapid tests for drug resistance on liquid media significantly reduced the time of waiting for the result to a few days, while obtaining results on solid media took about eight weeks. The application of molecular methods for the identification of drug resistance, based on the identification of gene mutations responsible for resistance to a drug, shortened the time to a few hours and, thus, contributed to a more rapid diagnosis of patients excreting drug-resistant strains, which is particularly important in patients with MDR and XDR tuberculosis.

XDR resistance was defined by WHO, in 2006, as MDR with resistance to fluoroquinolones and one of injectable drugs – amikacin and/or capreomycin. In 2013, XDR-TB incidence was reported to the World Health Organization by 100 countries. On average, it is estimated that 9.0% of people with MDR-TB have XDR-TB. Most of the cases were reported from Ukraine (1006), South Africa (612), India (364) and Kazakhstan (305). In the group of 1269 patients registered with XDR-TB in 2011, only 284 (22%) patients completed their treatment successfully, 438 (35%) died and there is no data on the rest.

One of the tests to quickly identify a patient with MDR-TB is a recently developed GenoType MTBDR plus test, detecting mutations associated with resistance to rifampicin and isoniazid among the strains of *Mycobacterium tuberculosis* complex.

The mechanism of action of rifampicin is inhibition of RNA polymerase at the β' subunit level encoded by the *rpoB* gene. RMP resistance is due to point mutations which give rise to altered β' subunit of RNA polymerase. This results in a lack of opportunity for a medication to bind to the enzyme and, as a consequence, inhibition of the life processes of bacterial cells (3, 4). Among the strains of *M. tuberculosis* resistant to rifampicin, the most often identified mutations are those in the *rpoB Ser531Leu*, *His526Asp* or *Tyr*, *Asp516Val* genes (5).

Isoniazid is a prodrug that, upon entering bacillus cel, is converted to an active form (isonicotinic acid) by the catalase-peroxidase enzyme encoded by the *katG* gene (6). The resulting isonicotinic acid forms, with the NAD+ or NADP+ coenzyme molecules, complexes that act as inhibitors of enzymes involved in the biosynthesis of nucleic acids and mycolic acids setting up the mycobacterial cell wall (6). Resistance to isoniazid is often associated with mutations in the promoter region of the *inhA*, gene encoding the enoyl-ACP reductase enzyme. This enzyme is essential for the mycobacterial cell to carry out a proper synthesis of mycolic acids and is sensitive to isoniazid. *InhA* gene mutation causes a change in the reductase structure, by which

it ceases to be sensitive to isoniazid and the enzyme does not lose its enzymatic activity. The mycobacterial cells, having a *inhA* gene mutation synthesis of mycolic acids, occurs in the presence of isoniazid.

It should, however, be noted that commercial molecular tests only take into account those areas of the genes responsible for drug resistance in which mutations occur most frequently. Therefore, it should be borne in mind that the drug resistance associated with mutations present in other places of genes are not identified by these tests, tuberculosis drug resistance may be due to other mechanisms, for example: the efflux pump.

AIM

The aim of this study was to compare the results of drug resistant strains of *Mycobacterium tuberculosis* complex obtained by phenotypic and molecular INH and RMP methods.

MATERIAL AND METHODS

Sixty strains of *M. tuberculosis* with different drug resistance were studied. In the case of all strains resistance test was performed for rifampicin, isoniazid, streptomycin and ethambutol in the Bactec MGIT 960 system and a molecular analysis of the GenoType MTBDR plus test.

The GenoType MTBDR plus test, using the DNA-STRIP method allows the identification of *Mycobacterium tuberculosis* complex strains and detection of resistance to rifampicin and isoniazid. Resistance to rifampicin is detected by identifying mutations in the *rpoB* gene, resistance to isoniazid – mutations in the *katG* and *inhA* genes. The procedure for identifying drug-resistant strains consists of three steps: isolation of DNA, amplification using primers labeled with biotin and a reverse hybridization. This hybridization reaction includes consecutive steps: chemical denaturation of the amplification products, hybridization of single-stranded amplicons labeled with biotin on a membrane coated with probes, washing, adding streptavidin/alkaline phosphatase conjugate, staining reaction using alkaline phosphatase. Strips stained on the test are identified in accordance with the identification model (fig. 1).

RESULTS

Among the 60 analyzed strains of *M. tuberculosis*, consistent results for rifampicin drug resistance in the conventional method of Bactec MGIT 960 and the GenoType MTBDR plus assay were obtained for 56 strains (93.3%). Three strains phenotypically resistant to RMP did not have mutations in the *rpoB* gene at positions 516, 526 and 531. In the case of isoniazid, results consistent in both methods were found in the case of 51 strains (85%). In the case of 5 strains, phenotypically resistant to INH, there were no mutation in the *katG* and *inhA* genes at positions: 315 (*katG*), -15, -16, -8 (*inhA*) (tab. 1).

Table 1. Comparison of the results of drug resistance to rifampicin and isoniazid, as obtained by the conventional method (Bactec MGIT 960) and the molecular GenoType MTBDR plus.

MTBDR plus molecular test		Bactec MGIT 960 phenotypic test	
		Resistant n (%)	Sensitive n (%)
Rifampicin	resistant	39 (65)	1 (1.6)
	sensitive	3 (5)	17 (28.3)
Isoniazid	resistant	46 (76.7)	4 (6.7)
	sensitive	5 (8.3)	5 (8.3)

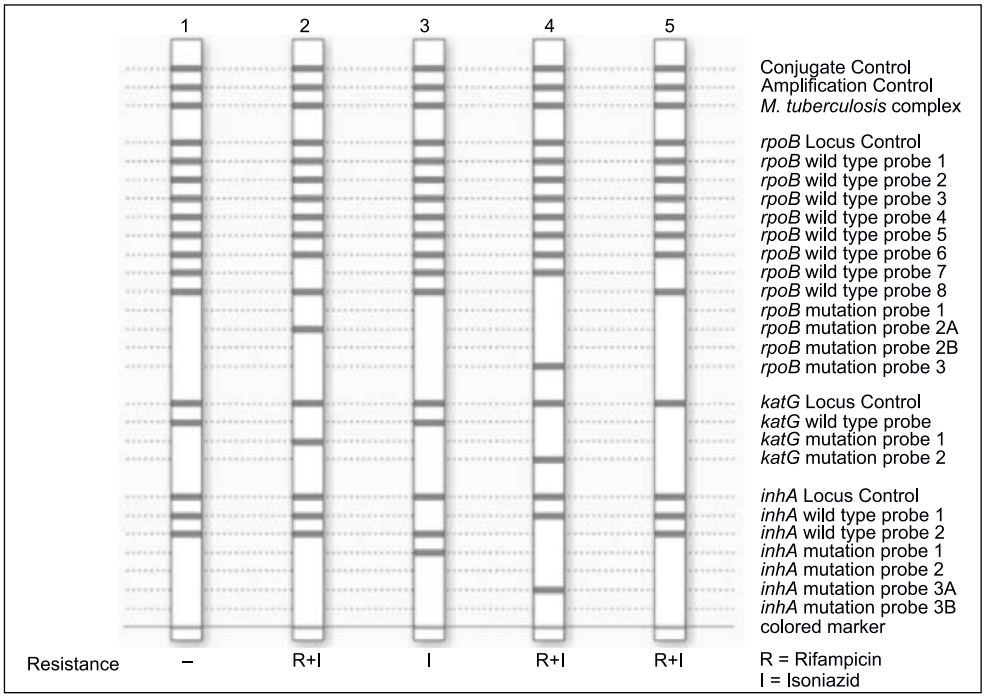

Fig. 1. Exemplified results of the GenoType MTBDR plus test.

Analysis of the results of drug resistance patterns (tab. 2), as obtained by two methods, showed the same pattern for 48 (80%) strains. Analyzing the pattern of MDR, consistent results in both methods were obtained for 34 (82.9%) strains. Results obtained for 7 strains were divergent. The molecular method did not identify resistance to RMP in 3 strains and resistance to INH in 4 strains. Divergent results also concerned four strains being phenotypically sensitive. Using the molecular method, an *inhA* gene mutation (position -15), conditioning isoniazid-resistance, was identified in the case of 3 strains. In one strain, mutations in *rpoB*, *katG* and *inhA* genes were identified – conditioning MDR.

Table 2. Comparison of drug resistance patterns obtained with the Bactec MGIT 960 phenotypic assay and the GenoType MTBDR plus.

MTBDR plus molecular test	Bactec MGIT 960 phenotypic test			
	MDR n	RMP – resistant n	INH – resistant N	Sensitive n
MDR	34	0	0	1
RMP – resistant	4	1	0	0
INH – resistant	3	0	9	3
	0	0	1	4

Among the 40 strains resistant to rifampicin, the molecular method identified *rpoB* gene mutations in 36 (90%). Analyzing the frequency of mutations, it was found that the S531L mutation prevailed – 29 strains (80.6%), 4 strains (11.1%) had H526D mutations, 2 strains (5.6%) – H526Y and in the case of one (2.8%) – it was the D516V mutation. Among the molecularly INH-resistant strains, in the case of 13, a mutation was identified in the C15T *inhA* gene, responsible for low titer of resistance. In the group of 39 strains in which, a mutation was identified in the *katG* gene, the S315T mutation was dominant and detected in the case of 36 (92.3%).

The GenoType MDR plus test sensitivity in the detection of resistance to rifampicin, isoniazid and MDR was respectively 92.9, 90.2, 82.9% at the assay specificity at 94.4, 55.5 and 94.7% (tab. 3).

Table 3. Sensitivity and specificity of the GenoType MTBDR plus.

	RMP	INH	RMP and INH
Sensitivity	92.9	90.2	82.9
Specificity	94.4	55.5	94.7
PPV	97.5	92	97.1
NPV	85	50	72

DISCUSSION

MDR-TB is one of the most serious threats to the health of the human population. While the non-resistant tuberculosis is a curable disease, during the standard 6-month treatment, MDR-TB requires very often 2 or more years, and only 50% of the new patients and 30% of the patients previously treated are complete cured. Excluding treatment failures, the mortality rate in this group of patients is also high, 43.7%. Apart from that, the treatment of tuberculosis caused by MDR mycobacteria is about 100 times more expensive and much more toxic.

Analysis of the incidence of drug-resistant TB is helpful in detecting and monitoring the spread of MDR and XDR strains and illustrates the effectiveness of tuberculosis surveillance in the country. As part of a properly conducted TB diagnosis, it is important to quickly identify a patient with drug-resistant tuberculosis. At present, identification of drug-resistant strains is possible by using genetic methods.

This paper presents a comparison of drug resistance test results performed by the phenotypic Bactec MGIT 960 and the molecular method using the GenoType MTBDR plus. Results of many studies of comparative tests for GenoType MTBDR plus in relation to conventional methods (Bactec 460Tb, Bactec MGIT 960) as well as other molecular tests (Inno-LiPA Rif.TB, sequencing) demonstrate their high sensitivity (11-15). In the case of our study, molecular test sensitivity to the detection of mutations responsible for resistance to rifampicin was 92.4%, which was comparable to the results of studies from Germany, Italy, Finland, France, Denmark, Turkey (92-100%) (11-13, 16-19). In the case of isoniazid, sensitivity of this test was set at 90.3% and correlated with drug resistance results obtained in Germany, Finland, Denmark and Taiwan (84-100%). A slightly smaller percentage (35-73%) of convergence between the results of the molecular and phenotypic tests detecting INH resistance was found in a study conducted in Turkey, Italy, France and the Caribbean (11-13, 16-20). The GenoType MTBDR plus test sensitivity in detecting MDR-TB strains in our study was 82.9%, which was slightly lower than the results obtained by other authors (91.1-98.8%) (14, 20).

Detection of resistance to rifampicin by the molecular method is based on the identification of the *rpoB* gene mutations. As shown in this study, among the strains phenotypically resistant to rifampicin, confirmation by the GenoType MTBDR plus molecular method was obtained for 94.4% of the strains. As evidenced by results of many studies (11, 16, 17), mutations in the *rpoB* codon 531 are the most frequent among the strains of *Mycobacterium tuberculosis* resistant to RMP. This is confirmed by the results of this study where, among the strains of *M. tuberculosis* resistant to rifampicin, the S531L mutation was found in 80.6% of the strains. Another mutation associated with resistance to RMP is the codon 526 mutation, the incidence of which is at the level of 3.2-44.8% (16, 17, 19, 21, 22). In the presented comparative analysis, 6 strains (16.6%) had a codon mutation. The least frequently identified was the D516V mutation in the *rpoB* gene (2.8%). The same results were obtained among drug-resistant strains in Spain and Denmark (11, 15).

Resistance of mycobacteria to isoniazid is primarily associated with katG Ser315Thr substitution (about

70% of INH-resistant strains) and C -15T mutation in the inhA promoter (15-35% of INH-resistant strains) (7-10).

In our study, a mutation at codon 315 of the gene *katG* concerned 72% of the strains of *Mycobacterium tuberculosis* complex. In the study conducted by Vijdea et al., it was demonstrated that all of the isolated strains of *Mycobacterium tuberculosis* complex (100%) from patients from Lithuania had the S315T mutation (11). In the previously published results, the incidence of this mutation is found at the level of 65 to 93.3% (16, 20, 23).

Mutations in the *inhA* gene of strains resistant to isoniazid are recorded less frequently; at the level of approximately 25%, which was also confirmed by the result of our study (26%) (9, 20, 21).

In countries with high TB incidence rates, among the isoniazid-resistant strains isolated from patients a high incidence of mutations in the *katG* gene is observed. Mutations in the *katG* gene are much less frequently identified in countries with lower rates of TB incidence rates and lower proportion of tuberculosis strains resistant to INH. Barnard et al., analyzing strains excreted by patients with drug-resistant tuberculosis, found differences in the prevalence of gene mutations responsible for resistance to isoniazid in the *inhA* and *katG* genes (20). It was related to the place of residence of patients with tuberculosis. Among the isoniazid-resistant strains isolated from patients in the province of KwaZulu Natal, the *katG* gene mutations were found in 97% of the strains and the *inhA* gene mutations in 24% of the strains. In the analysis conducted by Van Rie et al., it was found that INH-resistant strains isolated from patients with tuberculosis in the Western Cape Province in South Africa had *katG* mutations at the level of 72% and *inhA* mutations only in the case of 2% of the strains. Results of other researchers confirm differences in the incidence of individual mutations conditioning drug resistance (19, 23, 24).

It should, however, be noted that resistance to isoniazid is not only associated with the presence of mutations in the *inhA* and *katG* genes. In a study conducted by Hazbón et al., in 2006, a high percentage (about 25%) of INH-resistant strains was found, with none of the known mutations and, therefore, they were not identified as INH-drug resistant strains of molecular methods (25).

The short time-to-result and the possibility of administering correct treatment of the patient, according to the antibiogram, are obvious benefits of molecular tests. However, as shown by the results of numerous studies, tuberculosis drug resistance may be due to other gene mutations, not subject to the analysis of genetic testing. Therefore, further studies are needed on the improvement of molecular drug resistance testing, integration of new mutations into commercial testing, those responsible for resistance to antimycobacterial drugs. At the moment, in order to correctly identify drug resistance to antimycobacterial drugs, molecular diagnosis should include conventional drug resistance tests.

CONCLUSIONS

The MTBDR plus GenoType assay enables the identification of tuberculosis and identification of drug resistance to rifampicin and low and high titers of resistance to isoniazid. Since it is a new molecular test method, as recommended by the WHO, it should be used in parallel with the phenotypic method. Obtaining full correlation of both tests in their own country will replace the conventional method with the molecular.

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