Original Paper

Drug susceptibility testing of first-line anti-tuberculosis drugs: clinical evaluation of the differences between laboratories

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ABSTRACT

BACKGROUND: Tuberculosis (TB) remains a significant public health issue, partly due to drug resistance development. The rapid and reliable diagnosis is essential for TB control. The purpose of the present study was to detect and present the differences of molecular and phenotypic drug susceptibility testing (DST) of *M. tuberculosis* strains, isolated from patients hospitalized in the Pulmonary Department of the Aristotle University of Thessaloniki, as they were recorded by the Greek National Reference Laboratory for Mycobacteria (NRLM) (General Hospital for Chest Diseases "SOTIRIA", Athens, Greece) and the Laboratory for Tuberculosis (LT) of Northern Greece, G.H. "G. PAPANIKOLAOU". **METHODS:** Twenty-one TB patients were included. Culture of the Mycobacterium strain was conducted using Lowenstein-Jensen medium and phenotypic antibiograms were obtained from NRLM and LT. Molecular testing was conducted by NRLM. Isoniazid and rifampicin were tested. **RESULTS:** Eleven isolates yielded discrepant DST results. For isoniazid, 6 cases were found to be molecularly and phenotypically susceptible by NRLM while resistant by LT. In 3 cases, resistance was attributed to a problematic reagent. For rifampicin, four molecularly susceptible strains demonstrated phenotypic susceptibility at NRLM but resistance at LT. In one case, resistance was taken into account for treatment interventions. Furthermore, one strain demonstrated molecular and phenotypic resistance to rifampicin at NRLM, but susceptibility at LT. The strain was molecularly and phenotypically resistant to isoniazid and the patient was considered as a case of multidrug-resistant TB. **CONCLUSIONS:** Discordance between DST requires full consideration of the clinical presentation and collaboration with the laboratory and TB experts.
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INTRODUCTION

Tuberculosis (TB) remains one of the leading infectious diseases worldwide and therefore, it continues to be a significant public health issue, despite the fact that effective therapy is available, at least for the vast majority of patients. According to epidemiological data from the European Centre for Disease Prevention and Control (ECDC) more than 58.000 cases are notified annually in Europe[1]. In Greece, 519 cases were reported in 2014 (4.7/100.000 population). It is noteworthy that Greece is one of the few countries in the European Union with an increasing trend of TB incidence since 2010, while the increase concerns the native Greek population and not the foreigners[2]. Moreover, it is doubtful whether these data represent reality, due to the serious problem of TB under-reporting and to the constantly changing number of economic immigrants and refugees in the country[3-6].

Resistant TB is a global phenomenon distributed unequally among and within different countries. According to the latest World Health Organisation Global Report, in 2015 the estimated new cases of multi-resistant TB or TB resistant to rifampicin and the attributable deaths were 580.000 and 250.000, respectively[7]. Despite the fact that Greece is not one of the countries with a high TB incidence, the resistance rate is reported to be approximately 2.5%[2]. Resistant TB represents a therapeutic challenge because of the exceptionally long required treatment, along with the utilization of more expensive and toxic therapeutic regimens, which are related to higher rates of clinical failure and relapse[8,9]. Resistance to isoniazid is mainly due to mutations of the *katG* and *inhA* genes, while mutations of *rpoB* gene confer resistance to rifampicin. Diagnosis of resistance is performed by both phenotypic and molecular methods.

The rapid and reliable diagnosis of active TB is a prerequisite for TB control, especially in low-incidence countries[10]. The laboratory is required to play a decisive role both in individual cases and also more broadly at the level of epidemiological surveillance of resistance to anti-TB drugs[11]. The administration of the appropriate treatment depends entirely on the rapid and accurate detection of resistant and multi-drug resistant TB. Consequently and in order to ensure compliance with current international standards of laboratory diagnosis[12], all TB diagnostic laboratories in the European Union are required to acknowledge their technical proficiency and to have the performed tests accreditted by the official National Accreditation Body (in Greece this is the Hellenic Accreditation System or ESYD). Even when this is not possible, the establishment and operation in the laboratory of a Quality Management System that ensures at least appropriate infrastructure, equipment maintenance, staff training and participation in External Quality Assurance (EQA) Programs is considered essential for the laboratory's proficiency and the improvement of laboratory's efficiency in order to benefit patients and public health in general[13,14]. The introduction of new rapid methods for the detection of resistance mutations and the periodical participation in EQA Proficiency Testing Rounds, has lately highlighted problems associated with both specific genetic mechanisms and lack of standardization[15].

Phenotypic susceptibility testing is routinely performed in all primary anti-TB drugs and the required incubation time ranges between two and six weeks. However, the result of susceptibility testing is reported only in half of the bacteriologically confirmed cases in Greece[5]. Phenotypic testing is considered reliable for isoniazid and rifampicin with sensitivity and specificity between 97 and 99%[16]. Molecular susceptibility testing is a highly accurate diagnostic tool and it mainly includes two methods for the rapid diagnosis of resistance (Line Probe Assays and XPert MTB/RIF)[11,17]. Molecular assays decrease time to diagnosis from weeks to days, since they allow the detection of mycobacterial DNA or RNA directly from the patient's sample, before culture's becomes positive[18-20]. Rapid and accurate detection of resistance decisively influences the selection of the appropriate therapeutic regimen selection at an individual basis. Additionally, it has an impact on the epidemiological surveillance of the particular population[21]. Nevertheless, diverse resistance rates to primary anti-TB drugs have been reported among different laboratories, as well as among different diagnostic methods[8,22-24].

The purpose of the present study was to detect and present the differences of phenotypic and molecular susceptibility testing of *M. tuberculosis* strains, isolated from patients hospitalized in the Department of Pulmonary Medicine of the Aristotle University of Thessaloniki (General Hospital "G. PAPANIKOLAOU", Thessaloniki) as they are recorded by the Greek National Reference Laboratory for Mycobacteria (NRLM) (General Hospital for Chest Diseases "SOTIRIA", Athens) and the Laboratory for Tuberculosis of Northern Greece, G.H. "G. PAPANIKOLAOU".

PATIENTS AND METHODS

Twenty-one patients with pulmonary and extra-pul-

monary TB were included. All patients were followed up in the Department of Pulmonary Medicine of the Aristotle University of Thessaloniki, from August 2014 to June 2015. Culture of the Mycobacterium strain was conducted using solid Lowenstein-Jensen medium and phenotypic antibiograms were obtained from the Greek National Reference Laboratory for Mycobacteria (NRLM), "SOTIRIA" Hospital for Chest Diseases, Athens and the Laboratory for Tuberculosis of Northern Greece, G. H. "G. PAPANIKOLAOU" (LT) through applying the proportion method. The critical concentrations recommended by the latest revision of WHO were used[25]. Furthermore, molecular detection of mutations conferring resistance to anti-TB drugs was conducted exclusively by NRLM, due to the technical inability of the LMD laboratory to perform such techniques during the study period. Particularly, molecular testing of clinical specimens and/or strains was conducted through the Genotype MTBDR*plus* v.2 method (Hain Lifescience, Nehren, Germany). The results and the observed differences among the antibiograms regarding first-line anti-TB drugs isoniazid and rifampicin were recorded and consequently analysed.

RESULTS

M. tuberculosis strains were isolated from 21 patients, 13 men and 8 women, with a mean age of 50.67±20 years. Eleven patients had Greek nationality, 5 were from the Republic of Georgia and one from each of the following countries: Ukraine, Bulgaria, Pakistan, Russia and Moldova. Among the patients with concordant antibiograms, two had multi-resistant TB, while completely susceptible strains were isolated from the rest. In 9 cases, TB diagnosis was based on the detection of *M.tuberculosis* genetic material directly to the clinical sample, as the Ziehl-Neelsen acid fast staining microscopy was negative.

Differences between the two laboratories' antibiograms were observed in 11 of the 21 patients (Table 1). The differences found in those 11 patients are described and commented in Table 2. As for isoniazid, 6 cases were found to be molecularly and phenotypically susceptible by the NRLM while phenotypic resistance was reported at LT. In 2 of these cases, resistance found at LMD laboratory concerned only the critical concentration of isoniazid[25] (0.2 μg/ml), while in 4 cases it concerned both concentrations (0.2 and 1μg/ml). In 3 of these cases, the difference between the two laboratories was attributed to a problematic reagent used at the LT during that period. A replacement of the reagent was requested and after that the resistant rate to isoniazid was restored to normal rates. In two of those three patients, ethambutol was continued throughout the treatment period, while in the third, showing particularly extensive disease and slow progress, moxifloxacin and amikacin were added to the treatment regimen. Regarding the patient for whom the difference between the antibiograms was not attributed to the reagent's problem and showed resistance towards both concentrations of isoniazid according to LT, the worst case scenario was taken into account and isoniazid was replaced by moxifloxacin. For one of the two patients with resistance only to the critical concentration of isoniazid according to LT it was decided to remain on the conventional regimen due to the continuing excellent response, while the regimen was radically modified for the other patient due to the presence of significant side effects.

As for rifampicin, two molecularly susceptible strains demonstrated phenotypic susceptibility at NRLM, but resistance to both concentrations of 20 μg/ml and the critical of 40 μg/ml[25] at LT. No treatment was administered to one patient, as he stealthy discontinued his hospitalization, while the second one, showing extensive disease and slow progress, received additionally capreomycin and levofloxacin, taking into account the worst case scenario. For two patients, both molecular and phenotypic susceptibility were detected at the NRLM and resistance only to the concentration of 20μg/ml at LT. The above described difference has been observed quite often and according to our previous experience, it is not considered clinically significant, thus it did not lead to change of therapeutic regimens.

Furthermore, there was one patient with phenotypic resistance to isoniazid at both laboratories, phenotypic resistance to both concentrations of rifampicin at NRLM but phenotypic resistance to the concentration of 20 μg/ml and susceptibility to the concentration of 40 μg/ml of rifampicin at LT. Molecularly, this patient's strain demonstrated a ΔWT2 deficiency of the *rpoB gene* involved in resistance to rifampicin, and possible resistance to isoniazid due to another mutation (C(-15)T). This particular patient, who had received the conventional treatment regimen in the past, was considered as a case of multidrug-resistant TB and responded positively to a regimen including rifabutin, capreomycin, pyrazinamide, ethambutol and moxifloxacin.

DISCUSSION

A comparative evaluation of all the tested samples

TABLE 1. Drug susceptibility results from both laboratories.

Gender	Age	Ethnic Origin	NRLM Phenotypic Antibiogram				LT Phenotypic Antibiogram				Molecular testing	
			INH[a] 0.2[c]	INH 1	RMP[b] 20	RMP 40	INH 0.2	INH 1	RMP 20	RMP 40	INH	RMP
M	52	Bulgaria	R	S	S	S	R	S	R	R	C (-15)T	S
M	28	Ukraine	R	R	R	R	R	R	R	R	*S315T1*	*S531L*
M	27	Pakistan	S	S	S	S	R	R	S	S	S	S
M	51	Greece	R	S	R	R	R	S	R	S	C (-15)T	ΔWT2
M	62	Greece	S	S	S	S	R	R	S	S	S	S
M	40	Republic of Georgia	S	S	S	S	S	S	R	R	S	S
M	51	Greece	S	S	S	S	S	S	S	S	S	S
M	80	Greece	S	S	S	S	S	S	R	S	S	S
M	53	Republic of Georgia	S	S	S	S	R	S	S	S	S	S
M	55	Greece	S	S	S	S	S	S	S	S	S	S
M	74	Republic of Georgia	S	S	S	S	R	R	S	S	S	S
M	44	Greece	S	S	S	S	R	S	S	S	S	S
M	85	Greece	S	S	S	S	S	S	S	S	S	S
F	19	Republic of Georgia	R	R	R	R	R	R	R	R	S315T1	S531L
F	81	Greece	S	S	S	S	S	S	S	S	S	S
F	42	Russia	S	S	S	S	R	R	S	S	S	S
F	35	Moldova	S	S	S	S	S	S	S	S	S	S
F	74	Republic of Georgia	S	S	S	S	S	S	R	S	S	S
F	27	Greece	S	S	S	S	S	S	S	S	S	S
F	60	Greece	S	S	S	S	S	S	S	S	S	S
F	24	Greece	S	S	S	S	S	S	S	S	S	S

M: Male; F: Female; R: Resistance; S: Susceptibility

[a]INH, isoniazid; [b]RMP, rifampicin; [c]Drug concentrations are shown in μg/ml.

revealed significant differences between the antibiograms performed in the two laboratories. Differences concerned mainly the over-diagnosis of resistance both for isoniazid and for rifampicin from LT as compared to NRLM.

The identification of differences between the antibiograms results has been repeatedly reported in the literature. According to a recent study conducted by the Centres for Disease Control and Prevention (CDC) on 285 samples, concordance between phenotypic and molecular antibiogram was observed at a rate of 97.4% for rifampicin and 92.5% for isoniazid[21]. The differences observed were towards both directions, i.e. molecular resistance with phenotypic susceptibility or the other way around. However, when a strain was phenotypically susceptible to both drugs, absolute concordance of the methods was also evident. Most differences concerned stains susceptible to isoniazid by molecular testing, i.e. without *katG* and *inhA* mutations, for which phenotypic resistance was evident. The problem concerns mainly the commercially available CE/IVD molecular methods, as they do not cover the total of the genetic mutations involved in resistance to anti-TB drugs (approximately 98% coverage of the mutations to rifampicin and 85 to 90% of mutations to isoniazid)[11]. In cases with S531L mutation in *rpoB* gene and S315T1 mutation in *katG* gene phenotypic confirmation is certain. The mutation C (-15) T in *inhA* gene indicates low-level resistance to isoniazid· in this case the patient can theoretically receive higher doses of isoniazid, while in case of lack ΔWT2 in *rpoB* further investigation should be conducted applying sequencing approaches to determine whether the responsible mutation is «silent» or clinically important. Conversely, the clinician should be aware of the possibility of detecting mutations without clinical significance, as the «silent»

TABLE 2. Description of patients with discordant drug susceptibility testing.

Patient Gender, ethnic origin	NRLM	LT	Therapeutic intervention Comments
Isoniazid			
M, Republic of Georgia	Phenotypic&molecular susceptibility	Phenotypic resistance (critical concentration)	Therapeutic success with the classical first-line treatment regimen
F, Greece	Phenotypic & molecular susceptibility	Phenotypic resistance (critical concentration)	Modification of the therapeutic regimen due to side effects
M, Greece	Phenotypic & molecular susceptibility	Phenotypic resistance	Replacement of the reagent
M, Pakistan	Phenotypic & molecular susceptibility	Phenotypic resistance	Replacement of the reagent
M, Republic of Georgia	Phenotypic & molecular susceptibility	Phenotypic resistance	Replacement of the reagent
F, Russia	Phenotypic & molecular susceptibility	Phenotypic resistance	Replacement of isoniazid by moxifloxacin
Rifampicin			
M, Greece	Phenotypic & molecular susceptibility	Phenotypic resistance (low concentration)	No clinically significant-no intervention
F, Republic of Georgia	Phenotypic & molecular susceptibility	Phenotypic resistance (low concentration)	No clinically significant- no intervention
M, Bulgaria	Phenotypic & molecular susceptibility	Phenotypic resistance	No evidence
M, Republic of Georgia	Phenotypic & molecular susceptibility	Phenotypic resistance	Addition of capreomycin and levofloxacin
Multidrug-resistant Tuberculosis			
M, Greece			
Isoniazid	Phenotypic (low concentration) & possible molecular resistance	Phenotypic resistance (20 μg/ml)	Successful treatment regimen with rifabutin, capreomycin, pyrazinamide, ethamboutol and moxifloxacin
Rifampicin	Phenotypic &molecular resistance	Phenotypic resistance (20 μg/ml)	

M: Male; F: Female.

mutations are not a cause of resistance development and their detection should lead to dose modification of the respective anti-TB drugs[26]. Meanwhile, in 11 of the 180 samples of the CDC study for which phenotypic antibiogram was conducted in two laboratories (CDC and local), differences were found in isoniazid or rifampicin resistance profiles. These differences were attributed to the different methods and critical concentrations of the drugs used[21,23,24]. The differences observed were towards both directions, i.e. cases showing resistance according to CDC and susceptibility according to the local laboratory and vice versa. On the contrary, in our study, all cases of discordance were related to over-reporting of resistance (false positive results) from LT, while the opposite scenario was not observed. This phenomenon can be attributed to the different operational approaches between laboratories both in the US and internationally and in Greece, where clinical laboratories do not have accredited methods, do not carry out regular internal quality control programs and do not systematically participate in external EQA programs.

A possible explanation of the differences between the antibiograms could also be the co-infection with different *M. tuberculosis* strains, the significance of which has been reported in the literature. Current molecular methods allow the identification of different strains of the

same microbial species (heteroresistance-simultaneous presence of strains with different resistance profiles). More sensitive methods allow even the identification of the so-called minority strains, i.e. strains that constitute 1% of a sample's bacterial population[27]. According to a relatively recent review[28], the proportion of these patients with TB is far from insignificant (10-20%). This could lead to treatment failure after the implementation of the standard regimen[27,28]. According to the above, the presence of different strains could be one explanation of the differences between the antibiograms found in the present study. This could represent a possible scenario, since different sputum samples were sent to the two laboratories, which may also be derived from different regions of the lung parenchyma. Again, however, the very high percentage deviations approaching nearly 50% of the cases presented in this study, combined with the long-term relative experience in Greece indicating significantly lower heteroresistance rates, render this explanation rather weak.

The presence of differences among the antibiograms is an important issue as it has major clinical consequences. In the literature there are references of *M.tuberculosis* strains with *rpoB* mutations and a minimum inhibitory concentration (MIC) lower than the critical concentration of 1 μg/ml, currently used in phenotypic susceptibility testing ("low-level" resistance strains). The incidence of these strains was initially underestimated, probably due to lack of relevant studies[29]. Nevertheless, in the recent years their frequency and clinical relevance are becoming clearer, through studies correlating the presence of a mutation of the *rpoB* gene in a phenotypically susceptible strain with failure to the conventional regimen[29,30,31]. In a recent study conducted in Bangladesh and Congo including sputum of patients with TB and treatment failure or relapse, the rate of "disputed mutations" was estimated as greater than 10% of the total number of *rpoB* mutations[29]. At an epidemiological level, the genome analysis of multidrug and extremely drug-resistant strains revealed extremely high transmissibility of certain *rpoB* (516Tyr, 516Gly&533Pro) mutant strains[32]. Moreover, due to the phenotypic testing revealing susceptibility, diagnosis of resistance is quite delayed, leading to a long period of transmissibility with obvious clinical consequences for the general population[29]. It should be noted however that in Greece based on the data of NRLM, these mutations are extremely rare.

This study has important limitations. Despite the above described interpretations, it is true that the differences between the two laboratories were possibly more than expected. Unfortunately only one of the two laboratories that participated in our study (NRLM) has diagnostic tests of mycobacterial infections accredited by ESYD and has regularly participated in international EQA programs of WHO and INSTAND e.V. for the last eight years, has therefore proven technical proficiency to perform specific tests[14]. The second laboratory, the laboratory for Mycobacterial Diseases of G. H. "G. PAPANIKOLAOU", as probably many other laboratories of peripheral hospitals, operate without sufficient staff and the necessary equipment, making every effort with the minimum available resources to meet the increasingly growing needs of Northern Greece. As a result, samples are often sent from Northern Greece to NRML making diagnosis more complicated, time consuming and expensive. Clearly, for a more integrative laboratory support of TB diagnosis, laboratories with different levels of diagnostic capabilities should ideally exist in the Greek under the co-ordination of a reference center as parts of a single, organized network[33]. The small number of the examined strains, the possible objective differences in terms of sample processing between the two laboratories and the different clinical specimens tested in the two laboratories should also be included.

In any case, the differences between drug susceptibility testing of anti-TB drugs are a challenge for the clinician who is required to translate the sometimes conflicting information in treatment decisions. Therefore, both molecular and phenotypic antibiograms should be examined in total and cumulatively, while the clinical presentation of the patient should be taken into account for the final interpretation as indicated by experts in this field[34]. This was exactly the applied policy for the patients included in the present study. More specifically, in case of discordance between antibiograms in patients with extended disease and slow progress, it may be prudent to take into account the worst case scenario.

CONCLUSION

The differences identified on susceptibility testing of first-line anti-TB drugs between two laboratories are a real-life phenomenon already reported in the literature, with various explanations and clinical implications. In Greece, this phenomenon has some special features since the operation of the laboratories involved in the diagnosis of TB encounters significant problems regarding the harmonization of technical protocols, participation

in EQA programs, education and continuous training of the staff and finally accreditation of associated diagnostic tests. At the same time, major problems are the staffing and the equipment of peripheral laboratories. The clinician is requested to process the frequently conflicting laboratory data and make treatment decisions based on the patient's clinical presentation· always in collaboration with the laboratory and with experts in the treatment of TB.

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