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Reprint

Comparative efficiency of various molecular genetic methods in diagnosing tuberculosis

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Abstract: Objective – to compare the efficacy of various molecular genetic methods for diagnosing tuberculosis and determining drug susceptibility to rifampicin (RIF).
Materials and Methods. We conducted a retrospective study of the sputum analysis results on 1,992 patients with pulmonary tuberculosis treated at Saratov Oblast Clinical Tuberculosis (TB) Dispensary from 2014 through 2018. The following methods were used: real-time polymerase chain reaction (PCR), biological microarrays, automated Xpert® MTB/RIF technology. Statistical processing of the research results was carried out using the Bayes formula based on contingency tables (four-field table) and the $\chi 2$ test. When evaluating the significance of differences between relative values, we employed the critical significance level of 0.05.
Results. In terms of etiological diagnosis of TB, higher diagnostic sensitivities of the real-time PCR and biological microarray methods (73.9% and 70.3%, correspondingly) were established, as compared with the Xpert® MTB/RIF method (34.2%) ($p<0.001$). The sensitivity of all methods depended on the massiveness of bacterial excretion and clinical form of TB.
Conclusions: The Xpert MTB/RIF method exhibited lower diagnostic sensitivity in verifying the diagnosis of TB, whereas its operational characteristics in terms of determining RIF-resistance were sufficiently high (sensitivity at 89.7%, specificity at 89.1%, and efficacy at 89.4%), which was comparable with the characteristics of biological microarray method (93.9%, 71.8%, 82.9%; $p=0.127$, $p<0.001$, $p=0.139$, respectively).

Keywords: tuberculosis, molecular genetics, methods, diagnostics.

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Introduction

Currently, the tuberculosis (TB) remains among the most common infectious diseases both in Russia and worldwide. It is an important health care problem with significant economic and social consequences [1-3]. In modern phthisiology, a major role is assigned to the etiological diagnosis of TB, which means identifying the causative TB agent and determining its characteristics (drug susceptibility spectrum, species) [3-6]. These data are crucial for diagnosing, timely initiation of chemotherapy, choosing the correct treatment plan, monitoring an ongoing therapy, as well as implementing preventive measures in foci of TB infection [4, 6]. Traditional methods of inoculation on solid and liquid nutrient media still remain the gold standard for microbiological diagnosis of TB [6, 7]. However, the method of biological material inoculation on solid nutrient media is characterized by long wait for test results (2-3 months), and its efficacy in detecting mycobacteria is only about 60%. In recent years, there have been significant changes in the methodological base of clinical laboratory research to identify and characterize the causative TB agent [8]. Novel molecular genetic methods (MGM) have been developed for the etiological diagnosis of TB and for determining the drug susceptibility of the pathogen, which significantly increased the diagnosing efficacy and reduced the time of obtaining the results [4, 9-11]. At present, MGM are of particular importance due to the widespread multidrug-resistant TB both in Russia and worldwide [1] and are indispensable for the rapid determination of drug resistance (DR) and the choice of the optimal chemotherapy regimen. However, various types of MGM have been developed and approved for use in Russia: real-time polymerase chain reaction (PCR), DNA strip technology, biological microarray method, Xpert® MTB/RIF cartridge technology, etc.). All of these differ in information content, sensitivity and specificity of the assay. Hence, it seems relevant to conduct a comparative study of the efficacy of using various molecular genetic techniques in the practice of phthisiology.

Objective – to conduct a comparative analysis of the efficacy of using various MGM (specifically: real-time PCR, biological microarrays, and Xpert® MTB/RIF method) for diagnosing TB and determining drug susceptibility to rifampicin over a long-term follow-up period.

Materials and Methods

The design of our study was open, non-randomized, and diagnostic. A retrospective analysis of the results of sputum examination performed by different MGM (real-time PCR,

biological microarrays, Xpert® MTB/RIF) was carried out on 1,992 patients with newly diagnosed pulmonary TB who were hospitalized at Saratov Oblast Clinical TB Dispensary in 2014-2018. The research results were obtained by random sampling of data from medical records (inpatient case history and medical record of an outpatient TB patient – the forms 003/u and 081/u, respectively). Based on used MGM, the patients were divided into three groups, which were comparable in terms of the clinical forms of TB, the prevalence of the process, and bacterial excretion.

Group 1 consisted of 134 patients who underwent sputum examination using real-time PCR to confirm the diagnosis of TB. Real-time PCR is a modern high technology method, in which PCR products are detected directly in the course of amplification [10]. The studies were performed using a DNA amplifier with an optical unit for real-time PCR (iCycler iQ, Bio-Rad Laboratories, USA) and a reagent kit by DNA-Technology, Russia.

Group 2 included 1,417 patients who underwent sputum examination for the comprehensive diagnosis of TB via the Xpert® MTB/RIF method. Xpert® MTB/RIF is an automated cartridge-type PCR diagnostic system for the detection of Mycobacterium tuberculosis complex DNA and the rapid detection of DR to RIF with fully integrated sample processing. The studies were carried out using the GeneXpert® device and disposable cartridges (Cepheid, USA).

Group 3 consisted of 441 patients who underwent sputum examination by the biological microarray method to confirm the diagnosis of TB and determine the DR. The microarray method includes a two-stage multiplex PCR of gene loci, hybridization and registration of reaction products on a biological microarray, which allows detecting M. tuberculosis complex DNA and determining the DR of mycobacteria to isoniazid, rifampicin (RIF) and fluoroquinolones. The studies were carried out using the Chip-detector-01 computer appliance and the TB-Biochip test system, Biochip-IMB LLC, Russia.

In all three groups, the diagnostic sensitivity of the real-time PCR, Xpert® MTB/RIF and biological microarray methods was evaluated in a comparative aspect, depending on the clinical form of the TB process and the presence or absence of bacterial excretion in patients. In addition, a study of the efficacy of determining the drug susceptibility of mycobacteria to RIF was conducted on 294 patients selected from the total number of those examined. The diagnostic sensitivity (DS), diagnostic specificity (DSP) and diagnostic efficacy (DE) of the Xpert® MTB/RIF method (150 patients) and the biological microarray method (144 patients) were studied in comparison with the method of absolute concentrations after the inoculation on solid nutrient media.

Statistical data processing was carried out using Microsoft® Excel for Windows XP® and Statistica 6.0 software. To assess the diagnostic efficacy of the methods, the Bayes formula was used based on the contingency tables (four-field table). The $\chi 2$ test was performed to evaluate the significance of differences between the groups. When evaluating the significance of differences between relative values, we employed the critical significance level of 0.05.

Results

Comparing the results of real-time PCR, Xpert MTB/RIF and biological microarray studies, the highest amount of M. tuberculosis DNA was found in Group 1 in 99 of 134 patients with pulmonary TB. The DS of the real-time PCR method was 73.9%, which was significantly higher, compared with the Xpert MTB/RIF method (Group 2): 484 positive results out of 1,417 examined, which constituted the DS of 34.2%, p1-2<0.0001.

In Group 3 (bio-microarray method), 310 positive results were detected in 441 patients with pulmonary TB; hence, the DS of the method was 70.3%, which matched the real-time PCR sensitivity level of 73.9%. The data are presented in *Table 1*.

Table 1 data suggest that DS of employed methods depended on the massiveness of bacterial population in patients. The dominant number of positive results sensu all methods was obtained in patients with bacterial excretion, i.e., MTB(+): 87.5%, 45.6% and 79.3% in Group 1, Group 2 and Group 3, correspondingly. The highest sensitivity both in patients with bacterial excretion and in non-bacillary patients, denoted as MTB(–), was demonstrated by real-time PCR method (87.5 and 61.4%, respectively) and biological microarray method (79.3 and 57.8 %, respectively), which was significantly higher, compared with the Xpert MTB/RIF method (45.6% and 26.6%, respectively) (*Table 1*).

Additionally, we conducted a comparative study of the efficacy of these methods depending on the clinical form of pulmonary TB (*Table 2*).

In Group 1, the highest DS of the method was observed in patients with disseminated pulmonary TB (90%), whereas it ranged from 64 to 71% for different clinical forms. In Group 2, the highest DS was also recorded in patients with disseminated pulmonary TB, but overall, it was significantly lower compared with Group 1, and the range of fluctuations for all forms of TB was 27.5–48%.

Table 1. Comparative efficacy of various molecular genetic methods for the etiological diagnosis of tuberculosis

Patients with pulmonary tuberculosis	*M. tuberculosis* DNA detected / total number of examined subjects (%)			*p*
	Real-time PCR Group 1	Xpert MTB/RIF Group 2	Bio-microarrays Group 3	
MTB(+)	56/64 (87.5)	258/566 (45.6)	203/256 (79.3)	p_{1-2} <0.001 p_{1-3} =0.103 p_{2-3} <0.001
MTB(–)	43/70 (61.4)	226/851 (26.6)	107/185 (57.8)	p_{1-2} <0.001 p_{1-3} =0.563 p_{2-3} <0.001
Total	99/134 (73.9)	484/1417 (34.2)	310/441 (70.3)	p_{1-2} <0.001 p_{1-3} =0.504 p_{2-3} <0.001

MTB(+) – patients in whose sputum Mycobacterium tuberculosis were detected by any microbiological method; MTB(–) – patients in whose sputum Mycobacterium tuberculosis were not detected by any microbiological method.

Table 2. Comparative efficacy of various molecular genetic methods for diagnosing tuberculosis depending on its clinical form

Clinical forms of pulmonary tuberculosis	*M. tuberculosis* DNA detected / total number of examined subjects (%)			*p*
	Real-time PCR Group 1	Xpert MTB/RIF Group 2	Bio-microarrays Group 3	
Infiltrative	32/50 (64)	311/890 (35)	223/323 (69.1)	p_{1-2} <0.001 p_{1-3} =0.479 p_{2-3} <0.001
Disseminated	36/40 (90)	83/173 (48)	35/47 (74.5)	p_{1-2} <0.001 p_{1-3} =0.074 p_{2-3} =0.001
Tuberculomas	19/27 (70.4)	20 /99 (20)	23/31 (74.2)	p_{1-2} <0.001 p_{1-3} =0.799 p_{2-3} <0.001
Other (focal, cavernous and fibrous-cavernous, pleurisy, cirrhotic tuberculosis)	12/17 (70.6)	70/255 (27.5)	29/40 (72.5)	p_{1-2} <0.001 p_{1-3} =0.878 p_{2-3} <0.001
Total	99/134 (73.9)	484/1417 (34.2)	310/441 (70.3)	p_{1-2} <0.001 p_{1-3} =0.372 p_{2-3} <0.001

In Group 3, there were no significant differences in the DS method мы clinical forms of pulmonary TB: the range of fluctuations was detected at 69.1–74.5%. In general, in all clinical forms of TB, the DS of methods in Group 1 and Group 3 was significantly higher than in Group 2 (*Table 2*).

Next, we studied the efficacy of determining DR of M. tuberculosis to RIF via the Xpert® MTB/RIF method (150 patients) and the biological microarray method (144 people) in comparison with the method of absolute concentrations under inoculation on solid nutrient media. The results are presented in *Tables 3* and *4*.

DR of M. tuberculosis to RIF in the studied sample (n=150) was detected by the Xpert MTB/RIF method in 52 patients with TB (34.7%), and by inoculation on solid nutrient media in 58 patients of the same group: i.e., DS of the Xpert MTB/RIF method, in terms of determining DR to RIF, was 89.7%. In terms of determining DR of M. tuberculosis, DS is understood as the percentage of correctly identified drug-resistant strains of M. tuberculosis, and DSP is understood as the percentage of correctly identified drug-susceptible strains of M. tuberculosis against the reference value obtained by the cultural methods of research (inoculation on solid nutrient media). Inoculation on solid nutrient media yielded the growth of M. tuberculosis culture sensitive to RIF in 92 (61.3%) cases, while in the same patients, M. tuberculosis sensitive to RIF was detected by the Xpert MTB/RIF method in 82 people (54 .7%). Results agreement with the control for RIF susceptibility was found solely in 82 subjects, while in 10 cases there was no such agreement. Hence, DSP of the Xpert MTB/RIF method was 89.1% and the DE of the Xpert MTB/RIF method was 89.4%.

The results of a comparative analysis of determining DR of M. tuberculosis to RIF by the biological microarray method vs inoculation on solid nutrient media are presented in *Table 4*. Of 144 examined patients, DR to RIF was detected in 62 subjects (43.1%) by the biological microarray method vs 66 (45.8%) subjects by the inoculation method – that is, the DS of the bio-microarray method was 93.9%. M. tuberculosis drug susceptibility to RIF was detected by inoculation in 78 patients (54.2%), and by biological microarray method in 56 (38.9%) subjects. Accordingly, in 22 patients, mutations in the Rpo B gene were detected by biological microarray technology. Such mutations encoded DR to RIF, but were not confirmed by cultural studies. The DSP of the bio-microarray method was 71.8%, and its DE was 82.9%.

Table 3. Comparative results of determining resistance to rifampicin (RIF) by Xpert MTB/RIF assay vs absolute concentration method under inoculation on solid nutrient media

Laboratory method				Sensitivity of Xpert MTB/RIF (%)	Specificity of Xpert MTB/RIF (%)
Inoculation on solid nutrient media	*n*	Xpert MTB/RIF			
		RIF-resistant (number)	RIF-sensitive (number)		
RIF-resistant (absolute number)	58	52	6	89.7	89.1
RIF-sensitive (absolute number)	92	10	82		
Total	150	62	88		

Table 4. Comparative results of determining resistance to rifampicin (RIF) by biological microarray vs absolute concentration method under inoculation on solid nutrient media

Laboratory method				Sensitivity of biological microarrays (%)	Specificity of biological microarrays (%)
Inoculation on solid nutrient media	*n*	Biological microarrays			
		RIF-resistant (number)	RIF-sensitive (number)		
RIF-resistant (absolute number)	66	62	4	93.9	71.8
RIF-sensitive (absolute number)	78	22	56		
Total	144	84	60		

Discussion

At present, the microbiological diagnosis of TB enters a molecular genetics stage, which has promising prospects for solving the problems of faster detection of M. tuberculosis and determination of DR [4]. There are many different MGM and test systems on the market that have different operational characteristics and their own advantages and disadvantages. Health care practitioners experience significant difficulties in choosing the right research method and interpreting its results.

Our studies allowed identifying the features of various molecular genetics studies and determining more targeted indications for their use. E.g., our data on the highest sensitivity of the real-time PCR method in confirming the diagnosis of TB (73.9%) are similar to the results of our earlier studies [12], in which the DS of this method was 77.4%. Another highly sensitive technique is the biological microarray method: according to our data, it is not inferior to the real-time PCR, and its DS is comparable (70.3%).

The Xpert MTB/RIF method has insufficient sensitivity when examining patients for the etiological diagnosis of TB, its DS, according to our calculations, is 34.2%; hence, we believe that Xpert MTB/RIF can only be employed as a screening test, with subsequent use (in case of its negative result contradicting the TB clinical picture) of more informative MGM. However, it should be noted that while it has a low DS for confirming the diagnosis of TB, it is not inferior to the biological microarray method, in terms of determining the DR of M. tuberculosis to RIF, and its DS is 89.7% vs 93% in biological microarray method. DSP of determining DR to RIF for the Xpert MTB/RIF method is 89.1% vs 71.8% for the bio-microarray method. Both methods differ from culture studies (inoculation on solid nutrient media) in a higher percentage of detecting the DR to RIF. In our opinion, this is quite natural and understandable, since MGM are aimed at identifying the mutations in the M. tuberculosis Rpo B gene encoding the DR to RIF.

The emergence of genetic mutations is a factor preceding the formation of DR, detected by inoculation on solid media, and in the future, we should expect an increase in the number of gene mutations in such patients, which very soon would lead to the formation of DR to RIF. We believe that this also explains the lower specificity of the biological microarray method (71.8% vs 89.1% for the Xpert MTB/RIF method), since the bio-microarray technique detects 29 types of mutations in the Rpo B gene [4], while the Xpert MTB/RIF method detects just five most common types of mutations in this gene.

Conclusions

(1)A comparative analysis of the real-time PCR, Xpert MTB/RIF, and biological microarray methods showed that the real-time PCR and the biological microarray methods detected M. tuberculosis DNA in TB patients significantly more often (73.9 and 70.3%, respectively), compared with the Xpert MTB/RIF method (34.2%). (2) A higher sensitivity of real-time PCR and biological microarray methods, compared with the Xpert MTB/RIF method, was observed not only in patients with bacterial excretion (87.5 and 79.3 vs 45.6%, respectively), but also in oligobacterial patients (61 .4 and 57.8 versus 26.6%, respectively), in whom confirmation of the TB diagnosis by traditional microbiological methods was impossible. (3) The diagnostic sensitivity of MGM depended on the clinical form of TB and ranged from 64 to 90% in real-time PCR method, 20–48% in Xpert MTB/RIF method, and from 69.1–74.5% in the biological microarray method. (4) With a lower DS of the Xpert MTB/RIF method in the etiological verification of the TB diagnosis, when determining the DR to RIF, it exhibited sufficiently high operational characteristics (DS of 89.7%, DSP of 89.1%, DE of 89.4%), comparable with those of the bio-microarray method (DS of 93.9%, DSP of 71.8%, DE of 82.9%).

Conflict of interest: None declared.

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