

COMPILATION OF THE *MYCOBACTERIUM TUBERCULOSIS* BEIJING-B0 LINEAGE SAMPLE AND IDENTIFYING PREDICTORS OF IMMUNE DYSFUNCTION IN SOURCE PATIENTS

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Evolution of *Mycobacterium tuberculosis* have lead to the development of a number of lineages that have unique phenotypes and genotypes and are associated with certain geographical regions. Thus, compared to the reference strains of *M. tuberculosis*, Beijing and LAM genotypic lineages, which are the most common in the world, are highly virulent and transmissible. However, the extensive use of antibiotics over the past 50 years has caused the next evolutionary leap, which yielded new, epidemiologically dangerous sublineages: Beijing-B0 in Russia, Beijing-modern-4 in China and KZN in South Africa. This study aimed at investigating the effect the immune dysfunction predictors registered in patients have on the severity of tuberculosis (TB) developing after contracting *M. tuberculosis* Beijing-B0. We compiled a sample of patients with newly diagnosed TB caused by *M. tuberculosis* Beijing-B0, searched for the immune-suppressing diseases/conditions in their medical history and developed their immunograms. No connection was found between the state of the immune system and the characteristics of the disease we considered.

Keywords: *Mycobacterium tuberculosis*, virulence, drug resistance, compromised immune system, Beijing-B0/W148

Funding: the study is part of the project No. RFMEFI61317X0068 "The role of region-specific polymorphisms of virulence genes in the development of drug resistance by *Mycobacterium tuberculosis*" run by the Ministry of Science and Higher Education of the Russian Federation.

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Received: 02.06.2018 **Accepted:** 14.07.2018

DOI: 10.24075/brsmu.2018.040

СОЗДАНИЕ ВЫБОРКИ КЛИНИЧЕСКИХ ИЗОЛЯТОВ *MYCOBACTERIUM TUBERCULOSIS* ЛИНИИ BEIJING-B0 И ОПРЕДЕЛЕНИЕ ПРЕДИКТОРОВ ИММУННОЙ ДИСФУНКЦИИ ПАЦИЕНТОВ-ИСТОЧНИКОВ

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Эволюция *Mycobacterium tuberculosis* привела к появлению различных географически-ассоциированных линий бактерий, обладающих уникальными фенотипами и генотипами. Так, наиболее распространенные в мире генотипические линии Beijing и LAM проявляют высокий уровень вирулентности и трансмиссивности по сравнению с референтными штаммами *M. tuberculosis*. Однако за последние 50 лет, в результате массового применения антибиотиков, произошел очередной скачок эволюции, приведший к возникновению эпидемиологически опасных сублиний: Beijing-B0 в России, Beijing-modern-4 в Китае и KZN в ЮАР. Целью работы было исследование влияния предикторов иммунной дисфункции пациентов на тяжесть протекания туберкулезной инфекции при инфицировании *M. tuberculosis* Beijing-B0. Проводили отбор пациентов с впервые выявленным туберкулезом, вызванным *M. tuberculosis* Beijing-B0, анализировали анамнез каждого пациента-источника на предмет наличия заболеваний/состояний, вызывающих снижение иммунитета, а также определяли иммунограмму. В результате работы связи исследованных нами характеристик инфекционного процесса с состоянием иммунной системы пациента не обнаружено.

Ключевые слова: *Mycobacterium tuberculosis*, вирулентность, лекарственная устойчивость, иммунокомпрометация, Beijing-B0/W148

Финансирование: работа была выполнена в рамках проекта Министерства науки и Высшего образования России № RFMEFI61317X0068 «Роль регион-специфичных полиморфизмов генов вирулентности в формировании лекарственной устойчивости *Mycobacterium tuberculosis*».

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Статья получена: 02.06.2018 **Статья принята к печати:** 14.07.2018

DOI: 10.24075/vrgmu.2018.040

According to the World Health Organization, tuberculosis (TB) is one of the deadliest bacterial infectious diseases. In 2015, 10.4 million people contracted TB, including 1 million children. Up to 60% of all new cases are registered in India, China, South Africa (BRICS countries), Pakistan, Indonesia and Nigeria [1]. Especially dangerous are MDR (multi-drug resistant) and XDR (extensively drug resistant) strains of *M. tuberculosis*; the share of these TB pathogens is constantly growing. 45% of all new MDR strains are registered in India, China and Russia, which makes the situation in these countries particularly alarming [1–3].

The main methods of TB detection are chest radiography or fluorography, with MRI being the third option used less often. However, they can detect the disease only in the late stages. The other diagnostic methods are microbiological (selective plating media and subsequent microscopy) and molecular (PCR, mass spectrometry, ELISA- γ , lipoarabinomannan analysis etc) [4].

In addition to being drug resistance, *M. tuberculosis* is virulent, which makes it epidemiologically dangerous [5]. Strains of different phylogenetic lineages of *M. tuberculosis* were shown to have varying infecting ability. For example, Beijing strains are the most widespread and "successful" lineage and highly virulent, while those of the LAM-KZN phylogenetic lineage (peculiar to South Africa) tend to specifically affect people with immunodeficiency and cause death rapidly [6–8].

According to the preliminary estimates, Beijing-B0 isolates was detected in up to a half of the isolates from the first-time TB patients in Russia. The strains of this lineage are drug-resistant and highly virulent. To a certain degree, the same is true for the LAM-KZN lineage in South Africa and the Beijing-modern lineage in China. It should be noted that the three lineages mentioned above are very young: they appeared within the last 50–60 years, the age of antibiotics [2, 9]. There is a hypothesis that mutations in genes affected by antibiotics contribute both to the natural drug resistance and the associated virulence [3].

Thus, especially important are the studies uncovering the possibilities of preventing epidemics caused by "young" lineages of *M. tuberculosis*, as well as making anti-TB therapy more effective by detecting new, better adapted *M. tuberculosis* lineages through revealing the mutations associated with development of drug resistance and virulence [10]. This study aimed at analyzing the course of TB caused by *M. tuberculosis* Beijing-B0 in patients whose immune system offered varying levels of protective response. To attain the goal set, we collected and analyzed the *M. tuberculosis* clinical isolates while factoring in characteristics of clinical manifestation of the TB infection, as well as determined the degree to which the immune system of patients with "dangerous" forms of TB was compromised. In addition to the standard set of indicators for patients [11] and the data on *M. tuberculosis* drug resistance, it was necessary to take into account the patients' immune status.

METHODS

Bacterial strains and media

In the context of this study, we used the collection of *M. tuberculosis* clinical isolates of the Department of Microbiology and PCR Diagnostics of the National Medical Research Center for Phthisiopulmonology and Infectious Diseases (Ekaterinburg). Löwenstein–Jensen (LJ) and/or Novaya (BioMedia, Russia) media were used to cultivate the *M. tuberculosis* culture.

M. tuberculosis clinical isolates genotyping

Detecting the isolates belonging to the Beijing-B0/W148 genotype, we followed the applicable recommendations [2]. DNA isolation was carried out with the help of Proba NK sets (DNK-Tekhnologia, Russia), following the manufacturer's instructions. Isolated DNA were used for MIRU-VNTR genotyping done with TB-TEST commercial set of reagents (BIOCHIP-IMB, Russia), following the manufacturer's instructions. Amplification products were separated on 1.5% agarose gel, stained with ethidium bromide. Presence of the PCR product 1018 bp long indicated that the isolate belonged to the Beijing-B0/W148 genotype.

Estimating *M. tuberculosis* drug susceptibility

We applied the absolute concentration method to estimate the culture's susceptibility to anti-TB drugs: 0.2 ml of the suspension (containing 10 mln. bacterial cells) were plated into tubes containing solid LJ medium. The tubes medium also contained: no medicines (control); 1 $\mu\text{g/ml}$ of isoniazid; 40 $\mu\text{g/ml}$ of rifampicin; 2 $\mu\text{g/ml}$ of ethambutol; 30 $\mu\text{g/ml}$ of kanamycin, 30 $\mu\text{g/ml}$ of capreomycin; 1 $\mu\text{g/ml}$ of para-aminosalicylic acid; 30 $\mu\text{g/ml}$ of cycloserine; 30 $\mu\text{g/ml}$ of protionamide; 2 $\mu\text{g/ml}$ of ofloxacin. The *M. tuberculosis* culture was considered susceptible to the drug if the number of colonies developed did not exceed 20. When there were more than 20 colonies, the isolate was considered resistant.

Clinical isolates source patients

We used medical histories and results of peripheral blood tests of patients treated at the Ural Research Institute for Phthisiopulmonology (Ekaterinburg). The study was approved by the local ethics committee (minutes of the meeting No 59 of 14.11.2017); the data selected described adult patients who had TB diagnosed for the first time. All patients were divided into 2 groups: group 1 included patients whose immune system was compromised, group 2 — patients that had no conditions compromising the immune system. Group 1 ($n = 66$) inclusion criteria: *M. tuberculosis* Beijing-B0, hepatitis B (HBV), hepatitis C (HCV), human immunodeficiency virus (HIV), immunosuppressive syndrome (IDS), allergies, lymphoproliferative diseases, oncological diseases, rheumatoid arthritis, diabetes mellitus, chronic obstructive pulmonary disease (COPD); group 2 ($n = 34$) inclusion criteria: *M. tuberculosis* Beijing-B0, no immunocompromising conditions. Exclusion criteria: nonage, secondary tuberculosis.

Statistical analysis methods

Analyzing the data, we applied the chi-square test (χ^2) followed by a p-value calculation ($p < 0.05$). The χ^2 values were calculated in R software v 3.5.1.

RESULTS

Compiling a collection of clinical isolates

We compiled a sample of clinical isolates taken from TB patients in order to search for mutations in virulence genes that can be associated with drug resistance of *M. tuberculosis*. MIRU-VNTR genotyping allowed detecting whether the isolates belonged to the Beijing-B0 phylogenetic lineage. Profile analysis resulted in singling out 100 isolates of Beijing-B0/W148 genotype.

Each isolate was subjected to the drug susceptibility testing that made use of the absolute concentration method. All 100 isolates showed multiple drug resistance (MDR), i.e. resistance to rifampicin and isoniazid leastwise. 69 isolates were of the MDR+ phenotype (resistance to rifampicin, isoniazid plus resistance to fluoroquinolones or aminoglycosides/polypeptides).

Characteristics of clinical predictors of immune dysfunctions

In addition to determining drug susceptibility of the selected *M. tuberculosis* isolates, we have analyzed the source patient's immune system (compromised or not, compromising factors/degree) and disease pattern factoring in medical history and blood testing results. Some of the factors that define reversibility of the immune system dysfunction are starvation or deficiency of vital nutritional elements, metabolic diseases (diabetes mellitus, metabolic syndrome), mental depression and temporary distress of any nature. More severe immune system disorders can result from infections, ionizing radiation, lymphotoxic chemicals and lymphoproliferative diseases [12]. In the context of our study, we researched the predictors that are capable of stressing the immune system and keeping it in the stressed condition (Table 1).

Thus, tuberculosis can develop not solely after a contact with a TB patient but also following an endogenous scenario, i.e. activation of mycobacteria tuberculosis that have been in

the body for many years (latent infection). The patients were divided into two groups according to the status of their immune system: compromised or not.

Patients that suffered from both HIV and TB learned about their co-infection on average 37.5 ± 50.5 months from the date of their first diagnosis; the extremes of this term are 1 month and 13 years. In 3 patients that received antiretroviral therapy the level of viral load was undetectable. HIV patients had the viral load from not registrable to 1 million (0.22 ± 0.35 million) copies in 1 ml; the number of CD4 lymphocytes was from 148 to 1060 (611 ± 380) k/ml ($16.0 \pm 12.3\%$).

Clinical characteristics of TB infection

Generally, TB is known to develop in a body the immune system of which is compromised. In our study, there were twice as much patients with immune system compromising diseases than those without such (66 vs. 34 people). Despite the presence of clinical signs of immune deficiency, TB manifestations in both groups were much alike (Table 2).

In both groups, most patients had infiltrative form of tuberculosis. Disseminated form was somewhat less common in the first group, but the difference was insignificant ($p > 0.05$). Only the patients of the first group had extrapulmonary forms, which may be related to the compromised state of their immune systems. Table 3 presents the phases of TB infection in patients that participated in our study.

Table 1. Frequency of registration of clinical predictors of immune dysfunction in patients with compromised immune system

Nosology	Group 1 (n = 66)	
	n	%
HCV (hepatitis C virus)	23	34.8
HBV (hepatitis B virus)	4	6.1
HIV (human immunodeficiency virus)	14	21.2
Other manifestations of the infection	51	77.3
ID (immunodepression), allergic syndrome	5	7.5
Lymphoproliferative diseases (oncology)	2	3.0
Rheumatoid arthritis	1	1.5
Diabetes	10	15.1
COPD (Chronic Obstructive Pulmonary Disease)	14	21.2

Table 2. Clinical forms of TB in patients participating in the study

Clinical form of TB	Group 1 (n = 66)		Group 2 (n = 34)		χ^2	p
	n	%	n	%		
Infiltrative	39	59.1	20	58.8	0.003	0.955
Disseminated	5	7.6	3	8.8	0.083	0.773
Tuberculoma	14	21.2	6	17.6	0.003	0.955
Fibrous-cavernous	5	7.6	5	14.7	0.201	0.654
Extrapulmonary localization	3	4.5	0	0	0.361	0.548
Total:	66	100	34	100		

Table 3. Phases of TB infection in patients that participated in our study

Specific inflammation phase	Group 1 (n = 66)		Group 2 (n = 34)		χ^2	p
	n	%	n	%		
Infiltration	58	87.9	30	88.2	0.021	0.885
Degradation	41	62.1	25	73.5	0.145	0.704
Semination	29	43.9	15	4.1	0.698	0.403
Subsiding (compaction, resorption, calcification)	7	10.6	4	11.7	0.019	0.892

Chi-square was used to search for statistically significant differences between the groups. Results of the test given in Table 3 show that there is no significant difference in the incidence of specific inflammation between the groups ($p > 0.05$ for all groups).

Laboratory indicators characterizing state of the immune system

Along with clinical manifestations, there are some laboratory indicators that signal of the immune system dysfunction (Table 4). Deviations from standard values of such indicators allow assuming immune deficiency [13].

The number of neutrophils and monocytes that describes the phagocytic system function did not differ between the groups (Table 4). Analysis of the number of lymphocytes, which reflects the state of cell immunity, revealed no significant differences. Studying eosinophils, we noticed the standard deviation was above the average, which means there is a significant dissimilarity within the group. High dissimilarity leads to a suggestion that the first group patients had eosinophilia not only following an allergic reaction to medications, but also as a manifestation of concomitant allergopathology of parasitic invasion. At the same time, in the second group allergy to medications was the only reason, the response seen in any organism regardless of the immune system status. ESR level proved the groups did not differ in humoral component of the immune system.

The assumption about the humoral component of the immune system we made based on the ESR level (Table 4) was confirmed by the globulins concentration data (Table 5). This fraction reflects the number of immunoglobulins that determine

the level of this indicator. There were no significant differences in the synthesis of globulins in patients of the two groups. Glucose concentration levels were slightly heterogeneous in the first group because it included diabetes patients. At the same time, the second group was fairly homogeneous, which is proved by the small standard deviation value. Albumin synthesis and total protein levels did not differ in patients of the two groups.

DISCUSSION

To search for the peculiarities of TB caused by *M. tuberculosis* Beijing-B0 lineage, we compiled a sample of patients based on their medical histories, presence or absence of the immune dysfunction predictors (HBV and HCV, HIV, ID, allergies, lymphoproliferative diseases, oncological diseases, rheumatoid arthritis, diabetes mellitus, COPD), and tested their immune systems. Such a sample makes the analysis of the course of Beijing-B0-induced TB more detailed and high-quality; moreover, in the context of further comparative genomic studies it allows identifying the key markers (mutations) in *M. tuberculosis* isolates that make the strains especially dangerous to people with compromised immunity. It is crucial to factor in multiple indicators: lack of any piece of data on the source patient can make the results unreliable and the entire effort futile [14, 15]. The collection of 1000 isolates of *M. tuberculosis* compiled in Samara was not described in sufficient detail, which made continuation of the work impossible, thus proving the afore statement [11].

In addition to collecting the samples and describing the *M. tuberculosis* Beijing-B0 isolates and source patients, we analyzed the differences in characteristics of the infectious

Table 4. Characteristics of peripheral blood of patients that participated in the study

Indicators		Group 1 (n = 66)		Group 2 (n = 34)		95% CI	p
		T	σ	M	σ		
LEU		8.36	3.33	8.22	2.52	-1.134; 1.394	0.839
ESR		25.9	16.1	22.3	14.4	-2.32; 10.32	0.212
Rod neutrophils	%	3.6	5.1	2.3	4.2	-0.583; 3.383	0.165
Segmented neutrophils	%	59.6	11.7	56.9	12.1	-2.03; 7.63	0.253
	abs	5102	2588	4785	2076	-663; 1327	0.510
Eosinophils	%	2.5	2.2	4.4	5.9	-2.787; 0.050	0.736
	abs	206.6	204.1	392.4	678.1	-357; 11.98	0.823
Granulocytes	%	65.7	13.3	63.6	12.8	-2.993; 7.793	0.380
	abs	5674	2990	5384	2338	-821; 1464	0.579
Lymphocytes	%	25.6	12.5	28.1	12.7	-7.959; 2.359	0.284
	abs	1957	1015	2181	930	-651; 153	0.223
Monocytes	%	8.26	2.9	7.8	2.9	-0.665; 1.465	0.458
	abs	678	360	628	219	-86.07; 174.1	0.504

Note: M — average, σ — standard deviation, CI — confidence interval.

Table 5. Biochemical indicators of blood of patients participating the study

Indicators		Group 1 (n = 66)		Group 2 (n = 34)		95% CI	p
		M	σ	M	σ		
Glucose	Mmol/l	6.1	3.4	5.2	0.6	-0.318; 1.918	0.159
Albumins	g/l	40.7	6.7	40.1	8.5	-2.584; 3.384	0.791
Globulins	g/l	32.9	7.8	31.7	8.4	-1.954; 4.554	0.430
Albumin-globulin index	u.	1.3	0.4	1.3	0.4	-0.146; 0.146	1.000
Total protein	g/l	74.2	6.6	74.5	5.4	-3.134; 1.934	0.640

disease process and laboratory parameters between patients of the groups that differed in status of their immune systems; while the condition of this system at the outset of the TB development may be different, the clinical form of the latter has similar features. We did not find a significant effect of tuberculosis caused by *M. tuberculosis* Beijing-B0 on the clinical picture of the disease manifestation, as well as the connection of the immunogram indices of the patient, except in cases when significant differences in the character of the course of tuberculosis were found (only when the immune system was compromised significantly (eg, CD4⁺ lymphocytes less than 200 cells/ml)). To date, there is a number of studies published that demonstrated the specific danger (pathogenicity) of strains of this lineage at the molecular level [9, 10, 16] and on animal models [17, 18]. Probably, full genomic sequencing, analysis of mutation of virulence genes and pathogenicity will yield a clear answer to the question of "danger" of this phylogenetic lineage of *M. tuberculosis* and the connection to the status of the body's immune system.

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CONCLUSIONS

We have presented a sample of 100 clinical isolates of *M. tuberculosis* Beijing-B0, analyzed by drug resistance and source patient peculiarities. For each sample, we determined the immune system compromising conditions are built the immunogram. This approach seems to be key to high-quality genomic research aimed at combating the epidemic caused by a virulent and drug-resistant TB pathogen.

To date, there is no single form of registration of *M. tuberculosis* clinical isolates, especially in the context of genomic and phylogenetic studies. In this study, we have developed a "passport" for each isolate and completed it with data on the source patient. The data collected described status of the immune system, state of the patient's blood (immunogram), patient's medical history. The collection compiled can improve quality and scope of the future genomic research; it also simplifies the search for relationship between the patient's immune status and *M. tuberculosis* genotype.

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