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Peculiarities of TLR-2 (Arg753Gln) and TLR-4 (Asp299Gly) polymorphism prevalence in patients with acute brucellosis with cardiovascular lesions

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Abstract

Currently, there are single data on the relationship between TLR-2 polymorphisms (Arg753Gln) and TLR-4 (Asp299Gly) polymorphisms and susceptibility to brucellosis. Therefore, the aim of the study was to determine the frequency of TLR-2 (Arg753Gln) and TLR-4 (Asp299Gly) polymorphisms in patients with acute brucellosis with cardiovascular lesions in the Republic of Azerbaijan. 178 patients with a brucellosis clinic were examined. According to the criteria for inclusion in the study, only 120 people fully met all the criteria, which made up the main group. The control group consisted of 30 healthy individuals. TLR-2 (Arg753Gln) and TLR-4 (Asp299Gly) polymorphisms were also determined for all patients in both groups. In order to assess the state of the cardiovascular system, an electrocardiogram was recorded, blood pressure was measured, and an ultrasound scan of the heart was performed for all patients. It was found that 93 patients ($77.50 \pm 3.13\%$) with acute brucellosis had some or other signs of impairment in the work of the cardiovascular system, identified clinically or as a result of functional examination. Among carriers of the Asp/Gly genotype of the TLR-4 gene, an increased risk of brucellosis with CVS diseases was determined ($\chi^2 = 30.19$; $p < 0.0001$; OR = 24.29; 95% CI [5.45 - 108.37]), while the carriage

of the homozygous genotype Asp / Asp, by contrast, had a protective effect on the development of brucellosis (OR = 0.06, 95% CI [0.02 - 0.20]). Among the carriers of the Arg /Gln genotype and the Gln / Gln genotype of the TLR-2 gene, a significantly increased risk of brucellosis with CVS diseases was determined ($\chi^2 = 5.68$; $p = 0.02$; OR = 3.10; 95% CI [0.99 - 9.67]) and (OR = 2.48; 95% CI [0.53 - 11.61]), respectively. While the carriage of the homozygous Arg / Arg genotype, by contrast, was rarely seen in patients with brucellosis (OR = 0.28, 95% CI [0.10 - 0.74]).

Key words: acute brucellosis; TLR-4; TLR-2; polymorphism; cardiovascular system.

Introduction

Brucellosis is one of the most common zoonotic infections in the world, annually there are about 500 thousand new cases [1, 2]. Despite the wide spread of this infection in the world, there are areas with a pronounced endemic for brucellosis. These territories include the Republic of Azerbaijan, where new cases of brucellosis are recorded annually [3, 4].

An important social aspect of brucellosis is the high proportion among people with brucellosis - children and adolescents, people of young working age, as well as involvement in the epidemic process of people who are not professionally associated with the source of possible infection [5, 6]. The disease occurs with the affection of all organs and systems, which leads to the development of extremely adverse effects: loss of working ability and disability of patients, which determines its high socio-economic significance [7, 8]. According to different data, complications of the cardiovascular system in patients with brucellosis occur in less than 2-10% of patients. The endocardium is most often affected [9, 10].

It is known that the first line of protection of the body against infectious agents, including *Brucella*, is the innate immunity system [11, 12]. Microbial detection using Toll-like receptors (TLR) is one of the decisive steps in the activation of the innate immune response and is important for the reliable initiation of the adaptive immune response [13, 14]. Recent studies have shown that both members of the TLR family (TLR2 and TLR4) recognize components of different pathogens. Thus, TLR-2 interacts predominantly with lipoproteins of gram-positive bacteria, whereas TLR-4 interacts with lipopolysaccharide of gram-negative bacteria [15, 16].

To date, the study of the polymorphism of the genes of inflammatory regulatory molecules is of particular relevance. Determining their role in the pathogenesis of many diseases, including brucellosis, together with the achievements of modern genomics, allows

us to predict the risk of developing the pathology or the severity of its course, on the other hand, to personify pharmacotherapy.

Aim of the research

Determine the frequency of TLR-2 (Arg753Gln) and TLR-4 (Asp299Gly) polymorphisms in patients with acute brucellosis with cardiovascular lesions in the Republic of Azerbaijan.

Materials and methods

We examined 178 patients with brucellosis who sought medical care at the Baku Clinic and the Central Clinical Hospital of Baku. All patients gave permission for their inclusion in the study.

The diagnosis of acute brucellosis was established on the basis of clinical data, anamnesis, including epidemiological, objective examination data, results of laboratory diagnostics, including specific ones.

Specific research methods were performed by ELISA on Awareness and Stat Fax 3200 devices using NovaLisa Brusella IgG, IgM test systems (Germany) with the detection of IgM and IgG.

The criteria for inclusion in the study was the diagnosis of acute brucellosis, while taking into account the duration of the clinical symptoms, namely up to 3 months after the first complaints appeared.

According to the criteria for inclusion in the study, out of 178 patients examined, only 120 people fully met all the criteria, which made up the main group. The control group consisted of 30 healthy individuals who underwent a scheduled annual inspection. Groups were representative by age and sex. Patients of both groups are ethnic Azerbaijanis who permanently reside in the Republic of Azerbaijan. The average age of patients in the main group was 35.9 ± 2.8 years. Male subjects were predominant - 75.00%.

In order to assess the state of the cardiovascular system (CVS), all patients were registered with an electrocardiogram (ECG) according to the standard technique in 12 standard leads. Measurement of blood pressure (BP) was carried out according to the recommendations of WHO experts (2014). To assess the parameters of systemic and intracardiac hemodynamics, ultrasound test (EchoCG) of the heart was used using standard accesses for examination and analysis of the necessary indicators of the structural and functional state of the heart (as recommended by the European Society of Cardiology, 2016).

All patients were determined polymorphism (Arg753Gln) TLR-2. The DNA amplifications of the studied loci were carried out automatically using the following primer

structure: F: 5'-GAGTGGTGCAAGTATGAACTGGA-3', R: 5'-TCCCAACTAGACAAAGACTGGTCT-3'. After initial denaturation at 94 °C for 5 minutes, each stage was carried out in 3 stages: denaturation – 94 °C (30 s), annealing of primers (30-45 s), elongation – 72 °C (30-40 s). The final elongation lasted 10 minutes at 72 °C. To identify the alleles of the TLR-2 gene, restriction analysis of amplicons was performed using the Pst I restriction endonuclease (NPO 56) and restriction was performed for 12 hours at 37 °C. The products of hydrolysis of the amplified sequences were analyzed by electrophoresis in 2% agarose gel.

TLR-4 polymorphism (Asp299Gly) was also determined for all patients in both groups. Genomic DNA was isolated from peripheral venous blood, which was collected from the cubital vein into sterile vacutainers with stabilizer 44 (EDTA) using the “DNA / RNA isolation kit from serum or blood plasma” (NPF “LiTech”, Russia). The Asp299Gly polymorphism TLR-4 was amplified by PCR using specific oligonucleotide primers. Amplifications were performed on a Tertsik amplificator (DNA Technology, Moscow). The 25 µl PCR mix contained 2.5 µl of 10x amplification buffer; 2 mM magnesium chloride; 0.2 mmol of each dNTP; 66 ng specific primers; 2.5 units Act. Taq DNA polymerase (SibEnzyme, Russia); 20-50 ng of genomic DNA. 25 µl of mineral oil was layered into tubes. Annealing temperature for determining the Asp299Gly TLR-4 polymorphism site is 58 °C. TLR4 gene alleles were identified by restriction analysis of amplicons using the restriction endonuclease Bsp19 (SibEnzyme, Russia) at 37 °C. Cleavage products of the polymorphic portion of TLR4 genes were detected by electrophoresis in 3% agarose gel in 1 x TBE buffer (for 2 hours) at a voltage of 2V per 1 cm of gel). PBR322 / BsuRI was used as a DNA molecular weight marker. Gels were stained with ethidium bromide followed by visualization of the results in UV light.

The significance of differences in the distribution of genotypes in polymorphic loci between groups was checked for compliance with the Hardy-Weinberg equilibrium (http://gen-exp.ru/calculator_or.php).

Statistical processing of the research results was performed using the IBM SPSS Statistics program, version 12 (20) (licensed No. 9593869, belongs to the Department of Infectious Diseases with a course of epidemiology at the Vinnytsia National Medical University named after NI Pirogov of the Ministry of Health of Ukraine) using parametric and non-parametric assessment methods obtained results. The calculation of the arithmetic average value (M) and standard error of the indicator (m) was carried out. When assessing

quality indicators, the frequency of occurrence (%) and its standard error (m%) were calculated.

Comparison of the frequencies of genotypes and alleles was performed by analyzing the contingency tables using Fisher's exact test and χ^2 test, depending on the assumptions of the analysis. The risk of developing pathology was assessed by calculating the odds ratio (OR) with the 95% confidence interval (CI) determined using simple logistic regression.

Results and Discussion. The study found that 93 patients ($77.50 \pm 3.13\%$) with acute brucellosis had some or other signs of CVS malfunctioning that were identified clinically or as a result of a functional examination. Such a number of patients with changes in CVS, in our opinion, may be due not only to the direct action of the infectious agent, but also to the previously existing CVS lesion.

The most frequent clinical manifestations of CVS lesions were: weakening of heart sounds (in $45.83 \pm 3.73\%$ of patients), hypotension (in $40.83 \pm 3.68\%$ of people) and non-ischemic cardialgia (in $36.67 \pm 3.61\%$ of patients).

An analysis of ECG data in 12 derivations found that the overwhelming majority of pathologies concerned automatism disorders (in $67.54 \pm 3.51\%$ of patients) and ventricular repolarization processes (in $31.65 \pm 3.49\%$ of people). Moreover, among the disorders of automatism, the tendency to tachycardia dominated, the conduction disturbances were expressed mainly by the slowing down of impulse conduction along the bundle of His, and the excitability disturbances - mainly by the appearance of ventricular extrasystoles.

The data of echocardiographic examination of patients with acute brucellosis were also analyzed. In general, the average indicators of the structure and function of the heart in the group of patients with acute brucellosis, according to EchoCG, did not go beyond the framework of the corresponding generally accepted norms. However, the average values of individual indicators differed significantly from those in the control group. So in patients with acute brucellosis compared with the control group, an increase in heart rate was observed by 11.94% ($p < 0.05$), while the diastolic blood pressure was 18.77% lower ($p < 0.01$). The level of systolic pressure in the main group was not significantly different from the control group. Also in patients with brucellosis, significant differences in the size and volume of the left ventricle were found. Diastolic size and volume of the left ventricle in the main group, respectively, by 11.91% and 8.33% exceed those in the control group ($p < 0.05$). The size of the left atrium in the main group is 9.51% higher than the corresponding figure in the group of practically healthy individuals ($p < 0.05$). It has been established that the state of the systolic and diastolic functions of the left ventricle in patients with acute brucellosis varies

significantly. The main indicator of the systolic function of the left ventricle, namely the ejection fraction, is 6.90% lower in the main group of the study than in the control group ($p < 0.05$). However, a moderate decrease in left ventricular systolic function was recorded only in 6 patients (5.51%).

It was found that the Asp / Gly genotype of the TLR-4 gene was 12.7 times more frequent in patients with acute brucellosis with CVS than in healthy individuals and 9.5 times more often than in patients without CVS ($p < 0, 05$). Whereas, the Asp / Asp genotype was more often detected in healthy individuals. The mutant Gly allele was significantly more often, 5.2 times more common among patients with brucellosis with CVS lesion than among healthy individuals and 6.4 times more often than among patients with brucellosis without CVS lesion ($p < 0.05$) (**tab. 1**).

Table 1

The detection rate of polymorphism (Asp299Gly) of the TLR-4 gene in patients with brucellosis with and without CVS lesion

Genotypes and alleles TLR-4 (Asp299Gly)	Patients with brucellosis without CVS lesions (n=27)		Patients with brucellosis with CVS lesions (n=93)		Healthy persons (n=30)	
	Abs.	%	Abs.	%	Abs.	%
Genotype Asp/Asp	4	14.8±6.83*	31	33.3±4.88*	27	90.0±5.48
Genotype Asp/Gly	23	85.2±6.84*	59	63.5±4.99*	2	6.7±4.56
Genotype Gly/Gly	0	0	3	3.2±1.83	1	3.3±1.81
Allele Asp	31	57.4±9.52	121	65.1±4.94	56	93.3±4.56
Allele Gly	23	42.6±9.52*	65	34.9±4.94*	4	6.7±2.14

Note: * $p < 0.05$ - between patients with brucellosis with and without CVS diseases, and healthy individuals.

It was found that the Arg / Gln genotype of the TLR-2 gene was 2.4 times more frequently detected in patients with acute brucellosis with CVS lesion than in healthy individuals ($p < 0.05$). Whereas, the Arg / Arg genotype was more frequently detected in healthy individuals. The mutant Gln allele, significantly more often, 2.3 times was found among patients with brucellosis with CVS lesion than among healthy individuals ($p < 0.05$). Significant differences between the frequency of detection of polymorphism (Arg753Gln) of the TLR-2 gene in patients with brucellosis without CVS lesions and practically healthy individuals were not identified (**tab.2**).

Table 2

The frequency of detection of polymorphism (Arg753Gln) of the TLR-2 gene in patients with brucellosis with and without CVS lesions

Genotypes and alleles TLR-2 (Arg753Gln)	Patients with brucellosis without CVS lesions (n=27)		Patients with brucellosis with CVS lesions (n=93)		Healthy persons (n=30)	
	Abs.	%	Abs.	%	Abs.	%
Genotype Asp/Asp	20	74.1±8.43	49	52.7±5.18*	24	80.0 ± 7.3
Genotype Asp/Gly	4	14.8±6.83	30	32.3±4.85*	4	13.3±6.19
Genotype Gly/Gly	3	11.1±6.05	14	15.0±3.7*	2	6.7±2.96
Allele Asp	44	81.5±7.47	128	68.8±4.8	52	86.7±6.19
Allele Gly	10	18.5±7.47	58	31.2±4.8*	8	13.3±6.19

Note: * p <0.05 - between patients with brucellosis with and without CVS lesions, and healthy individuals.

The next step in our study was to establish the risks of brucellosis with CVS lesions, taking into account the carriage of polymorphic variants of the TLR-4 and TLR-2 genes. When studying the features of the distribution of allele frequency in patients with brucellosis, it was found that carriers of the Gly allele of the polymorphic (Asp299Gly) TLR-4 gene have an increased risk of developing brucellosis with CVS lesions (OR = 7.52, 95% CI [2.61 - 21.67]), then as in the case of carriage of the Asp allele, on the contrary, the risk of developing brucellosis is reduced (OR = 0.13, 95% CI [0.05–0.38]); the model is reliable at $\chi^2 = 17.98$, p <0.0001 (**tab. 3**).

Table 3

Association of alleles and genotypes for polymorphism (Asp299Gly) of the TLR-4 gene with susceptibility to brucellosis with CVS lesions

Genotypes and alleles	Patients with brucellosis with CVS lesions (n=93)	Healthy persons (n=30)	χ^2	p	OR	
					sign.	95% CI
Allele Asp	0.651	0.933	17.98	<0,0001	0.13	0.05 – 0.38
Allele Gly	0.349	0.067			7.52	2.61 – 21.67
Genotype Asp/Asp	0.333	0.900	30.19	<0,0001	0.06	0.02 – 0.20
Genotype Asp/Gly	0.634	0.067			24.29	5.45 – 108.37
Genotype Gly/Gly	0.032	0.033			0.97	0.10 – 9.66

We found that among carriers of the Asp / Gly genotype, a significantly increased risk of brucellosis with CVS lesions ($\chi^2 = 30.19$; p <0.0001; OR = 24.29; 95% CI [5.45 - 108.37])

was determined, whereas the carrier state is homozygous the Asp / Asp genotype, in contrast, had a protective effect on the development of brucellosis (OR = 0.06, 95% CI [0.02–0.20]).

When studying the characteristics of the distribution of allele frequency in patients with brucellosis, it has been found that carriers of the Gln allele of the polymorphic (Arg753Gln) TLR-2 gene have an increased risk of developing brucellosis with CVS (OR = 2.95, 95% CI [1.31 - 6.60]), model reliable at $\chi^2 = 7.36$, $p = 0.007$ (**tab. 4**).

Table 4

Association of alleles and genotypes for polymorphism (Arg753Gln) of the TLR-2 gene with susceptibility to brucellosis with CVS lesion

Genotypes and alleles	Patients with brucellosis with CVS lesions (n=93)	Healthy persons (n=30)	χ^2	p	OR	
					sign.	95% CI
Allele Asp	0.688	0.867	7.36	0.007	0.34	0.15 – 0.76
Allele Gly	0.312	0.133			2.95	1.31 – 6.60
Genotype Asp/Asp	0.527	0.800	5.68	0.02	0.28	0.10 – 0.74
Genotype Asp/Gly	0.323	0.133			3.10	0.99 – 9.67
Genotype Gly/Gly	0.151	0.067			2.48	0.53 – 11.61

We found that among patients with brucellosis with CVS and practically healthy individuals, the frequency of the heterozygous genotype Arg / Gln (Arg753Gln) of the TLR-2 gene was 0.323 and 0.133, respectively. That is, among carriers of this genotype, a significantly increased risk of brucellosis with CVS ($\chi^2 = 5.68$; $p = 0.02$; OR = 3.10; 95% CI [0.99 - 9.67]) was determined, while the homozygous genotype Arg / Arg, on the contrary, it had a protective effect on the development of brucellosis (OR = 0.28, 95% CI [0.10 - 0.74]). It was also found that carriers of the mutant homozygous genotype Gln / Gln also had an increased risk of developing acute brucellosis with CVS lesions ($\chi^2 = 5.68$; $p = 0.02$; OR = 2.48; 95% CI [0.53 - 11.61]).

The role of TLR-4 polymorphism and its relationship with brucellosis is an ambiguous and poorly understood problem. At the moment, in the available scientific literature, there are single data, as in one study, the polymorphism (Asp299Gly) of the TLR-4 gene was analyzed in patients with brucellosis. Based on the analysis of which, the researchers concluded that the Gly allele was more common among patients with brucellosis compared with healthy individuals (33.6% versus 20.7%, $p = 0.000003$). That is, this study first revealed the link between the genetic polymorphism of the TLR4 gene and susceptibility to brucellosis [17]. As

for the role of TLR-2 polymorphism and its relationship with brucellosis, at the moment there is only data relating to TLR-2 polymorphism and various diseases caused by infectious agents. So, it was found that the TLR2 polymorphism was associated with septic shock [18]. A link was also found with tuberculosis, rheumatic fever [19, 20].

Considering the small number of works devoted to the role of TLR-2 and TLR-4 polymorphism among patients with brucellosis, and especially certain groups with CVS lesions, of the hepatobiliary systems. In our opinion, an important aspect is a more thorough study of the state of the cardiovascular system with the obligatory use of instrumental methods of examination with the aim of early detection of signs of damage, timely involvement of appropriate therapeutic measures, as well as with the study of genetically determined risk factors for the development of these complications among patients with brucellosis.

Conclusions

1. The most frequent clinical manifestations of CVS lesions were: weakening of heart tones (in $45.83 \pm 3.73\%$ of patients), hypotension (in $40.83 \pm 3.68\%$ of people) and non-ischemic cardialgia (in $36.67 \pm 3, 61\%$ of patients).

2. The Asp / Gly genotype of the TLR-4 gene was 12.7 times more frequent in patients with acute brucellosis with CVS lesions than in healthy individuals and 9.5 times more often than in patients without CVS lesions ($p < 0.05$).

3. The Arg / Gln genotype of the TLR-2 gene was 2.4 times more frequently detected in patients with acute brucellosis with CVS lesions than in healthy individuals ($p < 0.05$).

4. Among carriers of the Asp / Gly genotype of the TLR-4 gene, an increased risk of brucellosis with CVS lesions ($\chi^2 = 30.19$; $p < 0.0001$; OR = 24.29; 95% CI [5.45 - 108.37]) was determined, while the carriage the homozygous genotype Asp / Asp, on the contrary, had a protective effect on the development of brucellosis (OR = 0.06, 95% CI [0.02-0.20]).

5. Among the carriers of the Arg / Gln genotype and the Gln / Gln genotype of the TLR-2 gene, a significantly increased risk of brucellosis with CVS lesions was determined ($\chi^2 = 5.68$; $p = 0.02$; OR = 3.10; 95% CI [0.99 - 9.67]) and (OR = 2.48; 95% CI [0.53 - 11.61]), respectively. While the carriage of the homozygous Arg / Arg genotype, on the contrary, had a protective effect on the development of brucellosis (OR = 0.28, 95% CI [0.10 - 0.74]).

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