==========METHODS==========

DEVELOPMENT OF MULTIPLEX REAL-TIME RT-PCR TO DETERMINE THE EXPRESSION LEVEL OF TOLL-LIKE RECEPTOR GENES

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Abstract. Immune response gene expression analysis is an important task in studies of interactions between host and an infectious agent. Many approaches to this task have been developed, but despite significant progress, the problem of selecting a single standard for data normalization remain unsolved. In the present work, *HPRT1*, *SDHA*, *GAPDH* and *TBP* were selected as candidates for reference genes with stable expression and a system for their analysis based on multiplex real-time RT-PCR was developed. The results of calculations using *geNorm* and *BestKeeper* algorithms allowed to create a stable index based on two genes – *HPRT1* and *SDHA.* It was used for normalization of expression level of target genes of Toll-like receptors: *TLR1*, *TLR2*, *TLR4*, *TLR6* and *TLR8*. The obtained expression values of Toll-like receptor genes in the sample of conditionally healthy individuals were characterized by high stability and positive mutual correlation (except *TLR6*), which may indicate common mechanisms of expression regulation and also confirms the possibility of using the developed multiplex system to analyze the expression of immune response genes.

Keywords: *Toll-like receptors, real-time RT-PCR, housekeeping genes, gene expression*

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INTRODUCTION

An important role in determining the role of genetic predisposition factors to multifactorial diseases is played by the features of the immune system's interaction with pathogenic microorganisms, which may be associated with the mechanisms of gene expression regulation.

Recognition of pathogen-associated molecular patterns (PAMPs) occurs through interaction with Toll-like receptors (TLRs) on the surface of immune system cells, leading to the activation of NF-kB, MyD88, and IRAK4 signaling pathways. It is known that the TLR4 receptor is responsible for the recognition of gram-negative bacteria (interacts with surface lipopolysaccharide), while heterodimeric complexes TLR1:TLR2 and TLR2:TLR6 are responsible for the recognition of gram-positive bacteria [1, 2]. The relationship between polymorphic variants of immune system genes and infectious diseases is being actively studied [3], however, in most cases, association studies only state the presence of a connection without revealing its nature.

Assessment of gene expression levels plays an important role in modern research. In the last decade, along with the development of massive parallel sequencing technology, the method of determining gene expression levels based on RNA-Seq has become widespread [4]. While having advantages over traditional PCR-based methods, RNA-Seq also has a number of disadvantages, including high cost and complexity, the need for bioinformatic processing of sequencing results, which makes the implementation of RNA-Seq in routine laboratory studies extremely difficult. In cases where there is no task of searching for all genes that differ in expression levels, but analysis of one or several genes is required, a method based on real-time PCR is more preferable [5, 6].

Multiplex real-time PCR with simultaneous reverse transcription in a single reaction mixture has several advantages, including high speed and simplicity of analysis, availability of reagents, and accurate real-time detection of results. Therefore, in this work, a method for determining the expression level of *TLR* genes was developed and tested using multiplex real-time PCR with reverse transcription (RT-PCR).

EXPERIMENTAL PART

Biological material. The study used 110 samples of whole venous blood obtained from conditionally healthy donors residing in Moscow and the Moscow region.

All procedures performed with human participants were in accordance with the ethical standards of the institutional and national research ethics committee and with the 1964 Helsinki

declaration and its later amendments or comparable ethical standards. Informed voluntary consent was obtained from each study participant.

Blood samples were collected in tubes with EDTA and stored at a temperature not exceeding +5°C. Nucleic acids were isolated using the "RIBO-prep" reagent kit after preliminary processing of samples with the "Hemolytic" reagent (FBUN CNIIE Rospotrebnadzor, Russia) according to the manufacturer's instructions. The isolated material was stored at -20°C for no more than a week until use.

Selection of genes . Housekeeping genes with stable expression in peripheral blood cells were used as reference genes: *HPRT1* (hypoxanthine-guanine phosphoribosyltransferase), *SDHA* (succinate dehydrogenase complex subunit A), *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *TBP* (transcription factor IID, TATA-box binding protein) [7–9] (https://www.ncbi.nlm.nih.gov/books/NBK143764/). Genes *TLR1* , *TLR2* , *TLR4* , *TLR6* and *TLR8* were selected as targets for expression determination.

Oligonucleotide design . When developing oligonucleotides, we followed standard requirements that we had previously used [10]. The concentrations of primers and probes, selected empirically, ranged from 0.2 to 0.4 and from 0.06 to 0.12 μM, respectively. To select the melting temperature and check for possible secondary structures (dimers, hairpins) of oligonucleotides, we used the idtDNA calculator (https://eu.idtdna.com/pages/tools/oligoanalyzer), and the specificity of primers to DNA and RNA templates was checked using the Primer-BLAST resource [11].

For each gene fragment, a pair of primers and a fluorescently labeled probe were selected. The primer sequences were chosen to be specific only to active splice variants characterized in the NCBI database. The design of oligonucleotides was performed to exclude DNA amplification either through annealing of one of the primers simultaneously in two adjacent exons (*a*), or through the mutual arrangement of primers in exons separated by an intron of several thousand nucleotide pairs (*b*), which prevents the production of DNA amplification product using TaqF polymerase.

The layout of oligonucleotides is shown in Fig. 1.

Fig. 1. Layout of oligonucleotides for detection of target regions and determination of gene expression levels. *a* – Annealing of one of the primers in two exons; *b* – the fragment size exceeds the possible processivity of the Taq polymerase used; I – DNA sequence; II – cDNA sequence.

Conditions for real-time RT-PCR . The reaction mixture (25 μl) contained the following components: PCR mixture with primers and probes (Table 1), containing dNTP (44 μM) – 1 μl, "RT-

PCR mixture 2 FEP/FRT" – 10 µl, "TaqF polymerase" – 0.5 µl, "Revertase (MMLV)" – 0.5 µl, "RT-G mix 1" – 0.25 µl, nucleic acid mixture diluted in "RNA buffer" – 10 µl. All reagents for nucleic acid extraction and RT-PCR were produced by the Central Research Institute of Epidemiology of Rospotrebnadzor.

The reaction was performed on a CFX96 amplifier ("BioRad", USA) according to the following program: 50°C for 15 min, 95°C – 10 min, then 45 amplification cycles: 95°C – 5 s, 60°C – 20 s, 72°C – 15 s.

To confirm the expected sizes of amplified fragments during RT-PCR, the absence of fragments when conducting the reaction without reverse transcription (negative control), and the absence of non-specific products (including dimers), electrophoresis of amplification products was performed in 1.7% agarose gel. The specificity of the amplified gene fragments was confirmed by sequencing a portion of the samples using the Sanger method with reagents and equipment from Applied Biosystems (USA).

The results of real-time RT-PCR (threshold cycle values (Ct)) were obtained using CFX Manager ™ ("BioRad") software.

Statistical analysis. Preliminary processing of real-time RT-PCR results was performed in Microsoft Excel. Samples with a positive signal in at least one of three replicates were selected. The Ct value matrix was exported to R environment (version 4.3.2) (https://www.R-project.org/). The average Ct values for each gene were calculated as the geometric mean (GM [Ct]) of independent replicates. To determine the distribution pattern of the analyzed values, the Shapiro-Wilk test and quantile-quantile plot construction were used. Assessment of intergenic relationships was conducted using pairwise correlation analysis, calculating the Pearson correlation coefficient (r) in the absence of statistically significant deviation from normal in each of the analyzed samples, and the Spearman correlation coefficient (*rho*) for significant deviations. The results of the statistical analysis were visualized as diagrams using the *ggplot2* package [12]. A value of $p < 0.05$ was used as the criterion for statistical significance of results. The Holm-Bonferroni correction was applied for multiple comparisons.

Analysis of housekeeping gene expression stability. The stability of housekeeping gene expression was determined using the algorithms *geNorm* [13] and *BestKeeper* [14], implemented in the *ctrlGene* (https://rdrr.io/cran/ctrlGene/) package. In *geNorm* the mean expression stability values (M) of the analyzed genes are calculated by stepwise exclusion of the least stably expressed genes in each round until only the most stably expressed genes remain (lowest M values). *BestKeeper* calculates descriptive statistics based on Ct data and pairwise correlation between all analyzed genes

taking into account the standard deviation (SD) of Ct values, where the lowest value represents the gene with the most stable expression in the ranking index. Based on the Ct values of the most stably expressed genes in each sample, *BestKeeper* indices were calculated and further used for normalization in the analysis of target gene expression.

Analysis of target gene expression. The ratio of the geometric mean of Ct values from independent replicates (GM [Ct]) to the *BestKeeper* index, calculated based on the most stable housekeeping genes, was used as a normalized indicator of target gene expression (Cq). To characterize the dispersion of Cq values in the analyzed sample, descriptive statistics and box plots were calculated for each gene.

RESEARCH RESULTS

Development of multiplex systems for expression level determination

Based on the analysis of published data, four genes with constant expression (for use as references) and five Toll-like receptor genes were selected, exon-intron structure was analyzed, and oligonucleotides for cDNA amplification were designed. All functionally active transcript variants described at the time of the study were taken into account.

Table 1 shows the oligonucleotide sequences obtained after system optimization and the sizes of PCR products. For the selection of fluorescently labeled probes, a region within the amplified locus was used. The choice was determined by comparative testing of primers and probes during method optimization.

Table 1. Oligonucleotides for gene expression determination

Gene, ID*	Primer	Nucleotide sequence (5′→3′)	PCR product length, bp
HPRT1, 3251	HPRT1-F	TTGCTTTCCTTGGTCAGGCA	97
	HPRT1-R	CCAACAAAGTCTGGCTTATATCCAAC	
	HPRT1-Z	(FAM)CGTGGGGTCCTTTTCACCAGCAAGC(BHQ2)	
SDHA, 6389	SDHA-F	GCGGCAACAGCAGACATGT	100
	SDHA-R	CTGTTTGCAACACTGTTGGCC	
	SDHA-Z	(R6G)CCGGGGCCTGTCGCGGCTGCTG(BHQ1)	
	GAPDH-F	GAAGGACTCATGCACCACAGTC	114

GAPDH, 2597	GAPDH-R GAPDH-Z	GCAGGGATGATGTTCTGGAGA (ROX)GCCCCGCGGCCATCACGCCACAG(BHQ)	
TBP, 6908	TBP-F TBP-R TBP-Z	GGTTTGCTGCGGTAATCATGA CAGTCTGGACTGTTCTTCACTCTTG (CY5)GAGAGCCACGAACCACGGCACTCATTTTC(BHQ2)	100
TLR1, 7096	TLR1-F TLR1-R TLR1-Z	TGGCAAAATGGAAGATGCTAGTC AGACTGCCAAATGGAACAGACAA (ROX)CCCGGAGGCAAT(BHQ1)GCTGCTGTTCAGCTC	297
TLR2, 7097	TLR2-F TLR2-R TLR2-Z	AGGCAGCGAGAAAGCGCAGC CCTTGGAGAGGCTGATGATGA (Cy5.5)CCCCCAAGACCCACACCATCCACAAAG(BHQ1)	230
TLR4, 7099	TLR4-F TLR4-R TLR4-Z	TTTCCCAGAACTGCAGGTGCTG CTCTGGATGGGGTTTCCTGTCA (R6G)GGGGCATATCAGAGCCTAAGCCACCTCTCTACC(BHQ1)	101
TLR6, 10333	TLR6-F TLR6-R TLR6-Z	ATGATGTTGCAGTGGCTATCCTAAAG CAACTCAGAGTTTTCACTCTTCCAAAG (Cy5)GGGTTGTTCTTCT(BHQ1)TCAGAGCATCTTGATATGAGTCC	97
TLR8, 51311	TLR8-F TLR8-R TLR8-Z	AGTTTCTCTTCTCGGCCACCT AGCAGGAAAATGCAGGTCAGC (FAM)CCTGCATAGAGGGTACCATTCTGCGCTGCTGC(BHQ1)	137

*Identification number in the Gene database (NCBI).

A total of eight gene expression product detection systems have been developed, with the expected size and composition of the PCR fragments corresponding to those obtained, which has been

confirmed by selective sequencing of amplification products. The developed mixtures of primers and fluorescently labeled probes are distributed between two reaction mixtures. The first reaction mixture (multiplex 1) contains a mixture of oligonucleotides that detect active splice variants of genes with constant expression: *HPRT1* , *SDHA* , *GAPDH* and *TBP* . The second (multiplex 2) contains a mixture of oligonucleotides for detecting expression products of *TLR* genes: *TLR1* , *TLR2* , *TLR4* , *TLR6* , *TLR8* .

The experimental scheme is shown in Fig. 2. Each sample was amplified in three replicates with reverse transcription and in three replicates without the reverse transcription step.

Fig. 2. Experimental scheme for determining expression levels in two multiplexes.

Performing amplification without reverse transcription did not lead to the formation of fragments in both multiplexes, while products of the expected length were obtained in reactions with the addition of reverse transcriptase.

Determination of the optimal combination of housekeeping genes

Comparison of GM [Ct] values revealed the highest expression level of the *GAPDH* gene with a mean value of 24.2, while the highest mean GM [Ct] value (lowest expression) was for *TBP* (31.2) (Fig. 3).

Fig. 3. Distribution of GM [Ct] values for housekeeping genes.

The lowest variation in GM [Ct] values is characteristic of genes *HPRT1* and *SDHA* (5.4 and 4.6 cycles respectively), while the largest (18.9) was observed for *GAPDH*. In general, the distribution of GM [Ct] values for all housekeeping genes did not significantly deviate from normal. Pairwise correlation analysis of GM [Ct] values (Fig. 4) also revealed the absence of statistically significant correlation between *GAPDH* and the pair *HPRT1* and *SDHA* and a low coefficient in the pair *GAPDH* and *TBP*. The highest correlation coefficient value of 0.75 was observed between genes *HPRT1* and *SDHA*.

Fig. 4. Diagram of pairwise correlations of GM [Ct] values of housekeeping genes. Statistically non-significant results are marked.

Calculation of expression stability using the *geNorm* algorithm showed that the pair *HPRT1* and *SDHA* is characterized by the highest stability – the lowest M value in which was 0.83 (*GAPDH* and *TBP* – 2.93 and 7.85 respectively). Taking into account the obtained results, stability analysis of three expression normalization indices *BestKeeper* – BK_4 based on all four genes, BK_3 – on three genes *HPRT1* , *SDHA* and *TBP* , and BK_2 – on two genes *HPRT1* and *SDHA* was conducted. All *BestKeeper* indices were compared both with each other and with indices calculated for the genes included in them (Table 2). A decrease in the variance of the *BestKeeper* index is observed even when including genes with the lowest stability.

Table 2. Descriptive statistics of Ct values for housekeeping genes (HKG) and indices calculated based on them *BestKeeper* (*BK*)

Parameter	*HPRT1*	*SDHA*	*GAPDH*	*TBP*	BK_4 ($n = 4$)	BK_3 ($n = 3$)	BK_2 ($n = 2$)
GM [Ct]	28.6	25.9	23.9	31.1	27.2	28.4	27.2
Min. [Ct]	26.3	23.9	13.9	26.5	23.3	25.6	25.2
Max. [Ct]	31.7	28.5	32.8	37.1	31.3	32.2	29.9
SD [±Ct]	0.9	0.9	3.5	2.3	1.5	1.2	0.8
CV [%Ct]	3.3	3.3	14.3	7.5	5.4	4.1	3.1

Note. SD [± Ct] – standard deviation of Ct; CV [% Ct] – coefficient of variation of Ct, %.

Based on the analysis results, the most stable index, BK_2, was selected for normalization of target gene expression values, with a standard deviation (SD) of less than one Ct cycle. The Pearson correlation coefficient between GM [Ct] of genes *HPRT1* and *SDHA* and the index BK_2 was 0.94 [95% CI = 0.89–0.96, $p < 0.001$] and 0.93 [95% CI = 0.89–0.96, $p < 0.001$] respectively.

Analysis of TLR Gene Expression Levels

Analysis of Ct values of *TLR* genes showed that *TLR6* is characterized by the lowest expression (GM [Ct] = 31.6) and on average "lags behind" the others by 4-5 cycles, while the differences between the averaged Ct values of the other genes did not exceed one cycle (Table 3) and were statistically significant (<0.05 with Bonferroni correction) in all pairwise comparisons, except for pairs *TLR1* - *TLR8* and *TLR2* - *TLR4* . No correlation was found between the GM [Ct] values of *TLR* genes and the BK_2 index, indicating differences in the expression patterns of housekeeping genes and *TLR* genes. The distribution of relative expression values (Cq) of *TLR* genes after transformation by division by the BK_2 index is presented in Fig. 5. Normalization reduced the dispersion of values for each gene but did not affect the overall distribution pattern and existing "outliers". The relative expression of *TLR6* is lower than that of other genes, which have mean expression levels close to those of housekeeping genes.

Table 3. Descriptive statistics of Ct values of *TLR* genes and their correlation with the BK_2

Parameter	*TLR8*	*TLR4*	*TLR1*	*TLR6*	*TLR2*
GM [Ct]	26.0	27.1	26.3	31.6	27.4
Min [Ct]	23.2	24.2	24.1	29.2	24.1
Max [Ct]	30.9	30.9	30.4	40.2	30.1
SD [±Ct]	1.12	1.28	1.23	0.95	1.00
CV [%Ct]	4.3	4.71	4.67	3.01	3.66
Correlation coefficient [*rho*]	0.34	0.37	0.32	0.10	0.23
p *	0.22	0.18	0.22	0.59	0.40

*With Holm-Bonferroni correction.

Fig. 5. Distribution of relative expression values (Cq) of *TLR* genes.

The pairwise correlation diagram between normalized gene expression values *TLR* is shown in Fig. 6. A statistically significant positive correlation is observed between the expression values of all *TLR* genes, except for *TLR6* , which has a weak correlation with *TLR4* and *TLR8* that does not reach statistical significance. The highest correlation was found for the relative expression (Cq) of the following gene pairs – *TLR4 – TLR8 rho* = 0.84 [95% CI = 0.70–0.92, p <0.001] and *TLR1 – TLR4 rho* = 0.79 [95% CI = 0.61–0.89, p <0.001] (Fig. 6). *TLR2* is characterized by a positive correlation

with the other *TLR* genes – from medium (with *TLR4* and *TLR8*) to high (with *TLR1* and *TLR6*) strength.

Fig. 6. Diagram of pairwise correlations of relative expression values (Cq) of *TLR* genes.

DISCUSSION OF RESULTS

The development of multiplex one-step systems for determining expression levels in the real-time RT-PCR format can be a non-trivial task, associated with the need for empirical selection of multiplex PCR conditions due to the large number of oligonucleotides simultaneously present in the reaction. In our work, RT-PCR techniques with five fluorescence detection channels were tested; in this case, the conditions were optimized for the simultaneous presence of 20 oligonucleotides in the reaction. In addition, before conducting a case-control analysis on different biological materials, it is important to select the optimal number of housekeeping genes for the most accurate determination of target gene expression levels.

The real-time RT-PCR method is widely used to determine gene expression levels, however, standardized approaches for evaluating analysis results of different types of biological material do not yet exist. For validation, methods based on determining the initial amount of cDNA and comparing with reference gene expression levels are most commonly used [15, 16]. In the present work, a methodology for determining gene expression in venous blood cells has been developed. The main features of this methodology are simultaneous detection of the expression of several target genes in one reaction mixture and performing reverse transcription, which makes it possible to carry out amplification without preliminary use of DNases to exclude amplification of isolated DNA. Simultaneous use of several targets for measuring expression levels in a single RT-PCR allows for optimal experimental planning by reducing the number of reaction mixtures and using RNA samples in equal concentrations for simultaneous quantitative analysis.

Since the main task is not to determine the exact amount of mRNA of target genes, but to establish the fact of a change in their expression level, reference genes with stable expression in the studied tissues are used for such a relative assessment. As a rule, housekeeping genes are used as reference genes, the expression of which does not change significantly during their life in cells and is practically not affected by external factors and experimental conditions. Nevertheless, the selection of suitable genes for normalization is problematic, since none of the available solutions is universal,

even within a single organism, the expression level of stable genes can vary significantly in different tissues. It is believed that the most reliable methods for analyzing gene expression should use at least two housekeeping genes [17, 18]. Most often, differential gene expression in peripheral blood samples is studied using the genes *HPRT1* , *SDHA* , *GAPDH* , *TBP* and *B2M* as references. At the same time, quite controversial results have been obtained when studying the expression of housekeeping genes *GAPDH* and *ACTB* in blood cells [17, 19, 20]. Our analysis of the stability of expression of four genes – *HPRT1* , *SDHA* , *GAPDH* , *TBP* – using *GeNorm* and *BestKeeper* showed that the most unstable of them is the *GAPDH* gene, which is confirmed by independent studies [21]. The results of the selection of reference genes showed that two housekeeping genes are sufficient for the formation of the *BestKeeper* normalization index, which is consistent with previously obtained results [22]. However, it is worth keeping in mind that the expression of reference genes in different tissues or cultured cell lines may be less stable. Thus, an independent sample showed that for the analysis of expression in peripheral blood samples, it is better to use other combinations of housekeeping genes than those selected in our work [23]. Such results may be associated both with the characteristics of the samples and with differences in the methods used.

The Ct values of *TLR* genes do not correlate with the Ct values of housekeeping genes, but some of the TLRs show high positive correlation of normalized expression values among themselves. Such interrelation suggests common mechanisms of expression regulation [24]. Experimental evidence shows increased expression and activation of TLR2 and TLR4 on alveolar macrophages and respiratory epithelial cells [25]. A high level of co-expression of *TLR1* and *TLR4* genes was also found in patients with chronic Takayasu arteritis, and *TLR4 – TLR6* in the control group. Interestingly, in the group of patients with active arteritis, such correlations were not detected [26]. Thus, changes in the expression level of *TLR* genes may be associated with both their regulatory features and the possible state of the donor's immune system at the time of biological material collection. The expression of *TLR* genes closely correlates with the characteristics of not only infectious diseases but also a number of oncological diseases. For example, the expression of *TLR1* , *TLR2* , *TLR4* and *TLR8* genes induces subsequent expression of *IL-6* and *IL-8* genes [27]. Changes in the expression level of *TLR* genes are associated with the inflammatory process and immune infiltration of lung tissues in multifactorial diseases [28]. Expression of *TLR3* , *TLR5* and *TLR9* is associated with certain subtypes of breast cancer, and the expression level of *TLR10* correlates with tumor stages, indicating the possibility of TLR influence on disease progression [29]. A significant increase in the expression of *TLR3* and *TLR4* at the early stage of squamous cell renal carcinoma was also found [30]. Thus,

changes in the expression level of *TLR* genes can be used as markers for both infectious and oncological diseases.

We have developed a multiplex system based on one-step RT-PCR, which has been successfully tested in analyzing the expression of Toll-like receptor genes in whole peripheral blood samples. Our results correlate with existing data and confirm the promising potential of using the developed system to study the association of changes in candidate gene expression levels with the risk of development and specific features of infectious diseases and related conditions. The use of four genes for expression level normalization allows the approach we present to be used for studying possible connections between immune response genes and various pathological conditions in samples from different tissues.

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ETHICS DECLARATION

All studies were conducted in accordance with the principles of biomedical ethics set forth in the 1964 Helsinki Declaration and subsequent amendments to it. They were also approved by the local ethics committee of the Central Research Institute of Epidemiology of Rospotrebnadzor of Russia, protocol No. 136 dated May 25, 2023.

Voluntary informed consent was obtained from each participant included in the study.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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