

METHODS

A Method for Evaluation of the Level of Circulating Mitochondrial DNA by *ND1* and *ND2* Genes

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The measurement of the level of mitochondrial DNA (mtDNA) in the blood is a difficult problem due to high variability of mitochondrial genes, deletions in the mitochondrial genome in some pathological conditions, different sources of mtDNA into the bloodstream (mtDNA from tissues, from blood cells, *etc.*). We designed primers and TaqMan probes for highly conserved regions of the *ND1* and *ND2* genes outside the mitochondrial deletions “hot zones”. For standardizing the technique, the true concentration of low-molecular-weight mtDNA was determined by real-time PCR for two targets: a fragment of the *ND2* gene (122 bp) and the *ND1* and *ND2* genes (1198 bp). The sensitivity and specificity of the developed approach were verified on a DNA pool isolated from the blood plasma of healthy donors of various nationalities. The concentration of low-molecular-weight mtDNA in the blood plasma of two patients with COVID-19 was monitored over two weeks of inpatient treatment. A significant increase in the content of low-molecular-weight mtDNA was observed during the first 5 days after hospitalization, followed by a drop to the level of healthy donors. The developed technique makes it possible to assess the blood level of low-molecular-weight mtDNA regardless of the quality of sampling and makes it possible to standardize this biological marker in a wide range of infectious and non-infectious pathologies.

Key Words: *circulating mitochondrial DNA (mtDNA); real-time PCR; COVID-19; ND1 and ND2 genes*

Mitochondrial DNA (mtDNA) is nucleic acids secreted by damaged or dying cells of parenchymal organs and blood cells. Free circulating mtDNA is included into molecular signals group, this pattern is recognized by type 9 toll-like receptors (TLR9) due to encoded hypomethylated CpG motifs resembling bacterial motifs [9]. Plasma concentration of mtDNA is increased

in patients with acute respiratory syndrome, multiple organ dysfunction during sepsis, trauma, hemorrhagic shock [2,5,7] and a number of other pathological conditions. In patients with COVID-19, high concentrations of mtDNA circulating in the blood are an unfavorable marker associated with the need for patient intubation and a high risk of death [6]. Mitochondrial dysfunction occurs in a wide range of pathologies such as Alzheimer's disease and cancer and during aging. Evaluation of mtDNA content can provide insight into the processes that either initiate or accompany various pathological conditions. For this purpose, the methods

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of reliable assay of the concentration of this biological marker are intensively developed [4]. However, there is a number of problems limiting the ability to assess mtDNA blood level: high variability of mitochondrial genes, deletion of certain parts of the mitochondrial genome in some tissues [4], different sources of circulating mtDNA (tissues and blood cells) [1]. The size of most DNA fragments freely circulating in human blood plasma does not exceed 300 bp [3], and less than 100 bp in the urine [8]. However, the main source of errors in assessing the level of circulating mtDNA in blood plasma most likely are errors at the preanalytical stage associated with improper use of vacuum blood collection systems, the absence of double centrifugation of corpuscles, *i.e.* “contamination” of low-molecular-weight mtDNA pool that has entered the bloodstream from affected organs with fragments of high-molecular-weight mtDNA released from destroyed blood cells. To solve the above problems, primers and TaqMan probes were designed for highly conserved regions of the *ND1* and *ND2* genes, which are outside the “hot zones” of mitochondrial deletions [4].

The purpose of the study was to standardize the PCR method for determining the concentration of circulating mtDNA calculated as the difference in the content of low-molecular-weight mtDNA (*ND2* gene fragment) relative to high-molecular-weight mtDNA (*ND1-ND2* genes).

MATERIALS AND METHODS

The study included 8 healthy donors of different nationalities (self-identification according to personal data): Yakuts (Sakha) (males subjects aged 31 and 47 years), Buryats (a male subject aged 69 years and a female subject aged 50 years), Mongols (a male subject aged 40 years and a female subject aged 29 years), and Russians (female subjects aged 52 and 70 years) and 2 COVID-19 patients (Russians, female subjects aged 62 and 45 years). The blood from the cubital vein collected by a vacuum system with EDTA as anticoagulant was used as the test material in all the

cases. To obtain plasma, the blood was centrifuged twice immediately after sampling at 1300g for 10 min at 4°C for sedimentation of blood cells. The plasma was repeatedly centrifuged in 1.5-ml plastic vials at 14,000g for 5 min at room temperature. The supernatant was transferred to clean vials, aliquoted, and stored at -80°C. DNA was extracted from 100 µl using DNeasy Blood Tissue Kits (Qiagen). Real-time PCR with primers (Eurogen) and TaqMan probes (Synthol) or SYBR Green qPCR Ready Mix (Sigma) solution was conducted for 45 cycles using AmpliTaq Gold 360 Master Mix reagents (Applied Biosystems) in the presence of 1× enhancer solution from the same kit on a CFX-96 cycler (Bio-Rad). Amplification protocol for low-molecular-weight mtDNA: 10 min at 95°C, polymerase activation; 15 sec at 95°C; 15 sec at 58°C; 15 sec at 72°C; for high-molecular-weight mtDNA: 10 min at 95°C, polymerase activation; 60 sec at 95°C; 15 sec at 57°C; 70 sec at 72°C.

Primers and probes (Table 1) were selected manually considering the variability data from human reference mitochondrial genome NC_012920.1 and the MITOMAP database (<https://www.mitomap.org/MITOMAP>).

Statistical processing of the results was performed using the Microsoft Excel 7.0 spreadsheet processor

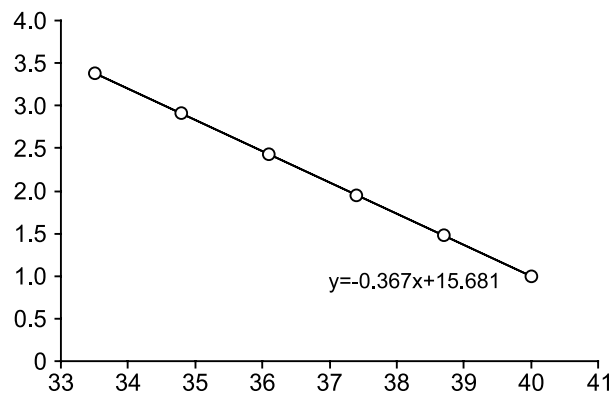


Fig. 1. Calibration curve. Ordinate: decimal logarithm of mtDNA concentration in genome equivalents in the samples obtained by successive 3-fold dilutions. Abscissa: threshold cycle Ct.

TABLE 1. Primers and Probes Used in the Study

Primers/ Probes	Sequence 5'→3'	Length, bp	Annealing, °C
Mt4463 F	CGTACTAATTAATCCCCTGGC (F)	122	58
Mt4585R	GCTAGCATGTTTATTTCTAGGC (R)		
Mt3440	[FAM]-GCGTCAGCGAAGGGTTGTAG[T-LNA]AG-[BHQ1]	1198	57
Mt3387 F	AAATTCTAGGCTATATACAACACTAC (F)		
Mt4585R	GCTAGCATGTTTATTTCTAGGC* (R)		

Note. *PCR visualization SYBR Green qPCR Ready Mix (Sigma).

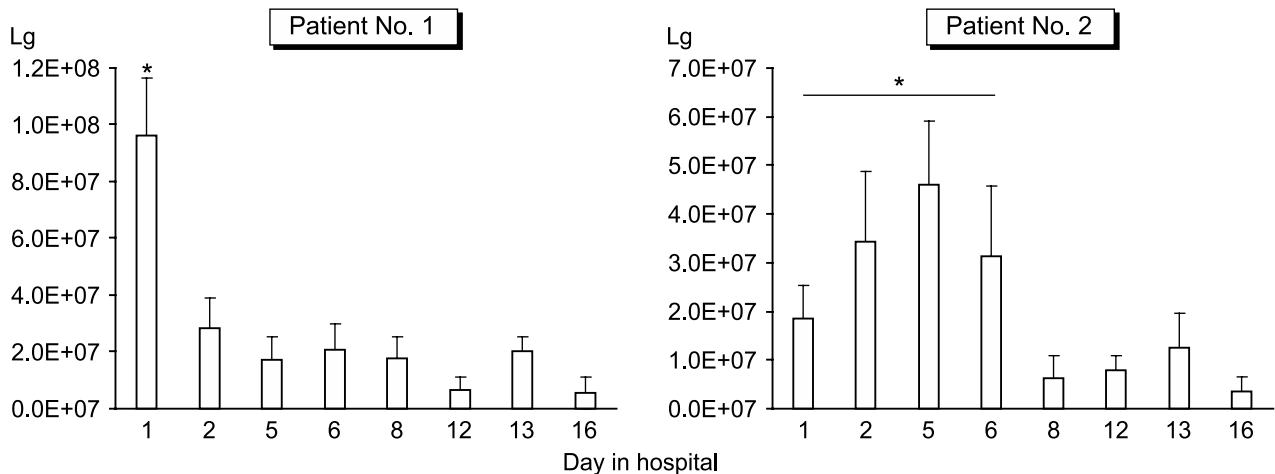


Fig. 2. Standardized content of mtDNA in the plasma of two COVID-19 patients. * $p \leq 0.05$ in comparison with subsequent days.

and Statistica 6.0 software (StatSoft, Inc.). The significance of difference between the values was estimated by ANOVA and Mann—Whitney U test. The difference was considered statistically significant at $p \leq 0.05$.

RESULTS

The calibration curve was constructed for pooled DNA sample from healthy donors of different nationalities (Fig. 1). The Ct value equal to 40 cycles was taken as 10 genome equivalents in the sample. The empirical equation $y = -0.367x + 15.681$ was used for further calculations of mtDNA concentration per 1 ml plasma.

To check the specificity of the developed tests, PCR tests were performed in triplicates with each of the 8 samples from subjects of different nationalities to assess the content of high- and low-molecular-weight mtDNA. The normalized value was calculated using the empirical formula (Fig. 1) per 1 ml plasma. True value of circulating low-molecular-weight mtDNA was calculated in genome equivalents as the difference between the concentration of low-molecular-weight mtDNA and high-molecular-weight mtDNA. The mean value equal to 5.6×10^6 genome equivalents of mtDNA per 1 ml donor plasma was obtained. However, in many test samples, high-molecular-weight mtDNA contamination exceeded 5%, *i.e.* was more than 2.8×10^5 . In other words, even the observance of all conditions at the preanalytical stage do not guarantee the presence of significant amounts of high-molecular-weight mtDNA extracted from blood cells during hemolysis, platelet lysis, *etc.*

A significant decrease in mtDNA concentration on day 2 (Patient No. 1) and on days 5-6 of treatment (Patient No. 2) was demonstrated. (Fig. 2). A common trend is observed; the difference in treatment days between the patients is probably determined by the delay in patient No. 2 hospitalization.

Thus, we have developed a real-time PCR-based molecular biological approach, which allows the use of circulating mtDNA level as a biological marker in healthy and infected people.

The authors declare no conflict of interest.

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