SHORT COMMUNICATION

Whole Blood Samples for Faster Real-Time PCR Analysis of Thrombophilic Mutations in SARS-CoV-2 Virus Positive Patients

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Summary

High incidence of thrombosis and venous thromboembolism was reported in patients with COVID-19. In this study, we focused on analysis of thrombophilic mutations performed without a standard DNA extraction step. In one hundred of COVID-19 positive outpatients, real-time PCR for Leiden mutation in the *FV* gene and G20210A mutation in the *FII* gene was carried out from DNA extracts and modified whole blood samples, and their cycle threshold (Ct) values were evaluated. In the extracts, healthy homozygotes (wt/wt), heterozygotes (M/wt), and homozygous carriers of Leiden mutation (M/M) provided median Ct values of 18.5, 19.4/22.0, and 20.9. In the whole blood, Ct values were 25.3 (wt/wt), 24.8/27.2 (M/wt), and 26.9 (M/M). Median Ct values for G20210A in the extracts were 19.6 for homozygotes (wt/wt), and 19.7/20.4 for heterozygous carriers. The whole blood samples provided Ct values of 23.9 in healthy homozygotes and 26.3/27.2 in heterozygotes for G20210A mutation. No homozygous subjects for G20210A and no double heterozygotes (for Leiden and G20210A mutations) were found. Despite significant differences in the Ct values, genotyping showed complete result concordance of the DNA extracts and the whole blood samples. The integrity and amplificability of DNA molecules in the whole blood samples during 28 days of deep freezing, interrupted by four cycles of thawing, did not significantly change. In conclusion, we demonstrated a new protocol for the detection of the thrombophilic mutations *via* real-time PCR on the modified whole blood of COVID-19 positive patients. The blood modification was reliable, easy, cheap, and saving costs and turnaround time of the whole laboratory process.

Key words

Real-time PCR • Whole blood • Thrombophilic mutation • SARS-CoV-2 virus • COVID-19

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is responsible for the ongoing coronavirus disease 2019 (COVID-19) pandemic that has resulted in more than 6.1 million deaths in the world [1]. A substantial part of patients suffering from COVID-19 develops severe complications like acute respiratory failure, septic shock, heart failure, and hepatocellular and kidney injuries. In intensive care units, high incidence of thrombosis and venous thromboembolism were particularly found in cases with poor prognosis [2]. Unusual elevation of factor V activity >200 IU/dl in COVID-19 patients was subsequently reported [3].

Factor V Leiden mutation (c.1691G>A; p.R506Q; rs6025) and G20210A mutation (c.20210G>A, rs1799963) in the *FII* gene play important roles in the coagulation cascade. Their carriers have an increased risk of deep venous thrombosis or pulmonary embolism [4-6]. The presence of Leiden mutation was reported to potentially impair clinical symptoms of developing COVID-19 coagulopathy [7]. For that, fast laboratory analysis of thrombophilic mutations could contribute to reveal inherited risk factors for development of this complication.

Determination of Leiden and G20210A mutations is mainly performed *via* certified *in vitro* diagnostics (IVD) kits based on real-time PCR and technology of TaqMan hydrolytic probes. Considering shortening the laboratory process in COVID-19 patients for fast evaluation of their clinical state and decision of the optimal treatment regimen, in this study we focused on analysis of these mutations performed without a standard DNA extraction step. Using whole blood samples for the analysis *via* conventional techniques like amplification refractory mutation system, semi-nested PCR, restriction fragment length polymorphism, or single strand conformation polymorphism was previously reported [8-11]. Later, Castley *et al.* used formamide-treated blood for real-time PCR with hybridization probes followed by melting curve analysis [12]. To our best knowledge, there is only one recent publication dealing with analysis of thrombophilic mutations performed with TaqMan probes chemistry and using whole blood samples [13]. Finally, the technology of loop-mediated isothermal amplification (LAMP) for these mutations without prior DNA extraction was published, as well [14]. In this study we present a simple modification of whole blood samples carried out before real-time PCR ensuring faster, easier and cheaper detection of both the mutations in COVID-19 patients.

The experimental group consisted of one hundred of COVID-19 positive outpatients (53 females and 47 males with a median age of 37 years; range 18-77 years) of Charles University Hospital in Hradec Králové, Czech Republic. Their COVID-19 positivity was determined *via* RT-PCR analysis [15]. Patients using therapeutic anticoagulation were excluded from the study. All subjects gave their informed consent for the genetic testing before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Charles University Hospital in Hradec Králové, Czech Republic (project identification code: COVID-19 FN HK Project A, reference number 202005 S01P, date 20 April 2020).

3 ml of venous blood with EDTA (BD Vacutainer sampling tubes) was collected from each subject at diagnosis and stored at -70 °C until analysis. Repeated thawing and freezing was avoided. 20 µl of the blood sample was diluted with 20 µl of deionized water and incubated at 90 °C for 3 min. After that, the samples were spinned at 10000× g for 2 min and the resulting supernatant was immediately used for PCR analysis. In the case of extracts, genomic DNA was isolated from 200 µl of the same blood sample as above by the MagCore Genomic DNA Whole Blood Kit (RBC Bioscience, Taiwan) according to the manufacturer's instructions. The final elution volume was 200 µl.

Concentrations of DNA molecules in the supernatants and extracts were determined *via* their amplificability in the gb Genetic Human DNA Assay (Generi Biotech, Czech Republic) in a Rotor-Gene 6000 (Corbett Research, Australia). The amplification primers and hydrolytic probes for real-time PCR were located in intron 1 of the *POLR2A* gene (chromosome location 17p13.1). The assay amplified 86 base pair (bp) products. Using ten-fold serial dilutions of the Generi Biotech Standard Human Positive Control DNA (20 ng/µl), a calibration curve ranging from 0.002 ng/µl to 20 ng/µl was constructed.

The mutations were determined by the gb HEMO FV Kit and gb HEMO FII (G20210A) Kit (both Generi Biotech, Czech Republic) approved for *in vitro* diagnostics (CE-IVD certification), subsequently verified and accredited for clinical use by the Czech Institute for Accreditation. These assays (serving in this study as a gold standard) work on the principle of allelic discrimination performed by real-time PCR with fluorescent hydrolytic probes in the Rotor-Gene 6000. For analysis, 4 µl of each DNA extract or each obtained supernatant was added into the PCR mixture (16 µl). In real-time PCR, amplicons of 110 bp were formed and their cycle threshold (Ct) values were evaluated (Fig. 1). The threshold was set to 0.025. A verified working range of these assays was 0.01-35 ng/µl, which corresponded with Ct values lying between 20 and 33. Stability of DNA molecules in the whole blood samples stored at -70 °C was tested by the gb HEMO FV Kit for 28 days. Each week, the storage period was interrupted by thawing the tubes to the room temperature and performing real-time PCR. Obtained Ct values were evaluated as above.

All experiments were done in duplicate, and the mean Ct value for each sample was calculated. Based on the Anderson-Darling test for data distribution, the parametric or nonparametric test was used to ensure test sensitivity. Differences between the whole blood supernatants and the DNA extracts were assessed using the Student's *t*-test or the Wilcoxon rank-sum test. $P<0.05$ were considered statistically significant.

Quantitative real-time PCR analysis performed in the extracts revealed a median DNA concentration of 18 ng/µl (range 2-29 ng/µl) and a median yield of 3.6 µg (0.4-5.8 µg). Table 1 shows Ct values in the extracts obtained in Leiden mutation analysis. For healthy homozygotes (wt/wt), heterozygotes (M/wt), and homozygous carriers of the mutation (M/M), median Ct values of 18.5, 19.4/22.0, and 20.9, respectively, were recorded. Median Ct values for G20210A were as follows: 19.6 for homozygotes (wt/wt), and 19.7/20.4 for heterozygous carriers.

Using the same quantifying kit as above, the number of DNA molecules present in modified whole blood samples was determined, as well. A median DNA concentration of 0.2 ng/µl (range 0.06-4.3 ng/µl) was revealed. Then, PCR analysis for the thrombophilic mutations followed. Ct values of 25.3, 24.8/27.2, and 26.9 in healthy homozygotes, heterozygotes, and homozygous carriers of Leiden mutation were obtained. Similarly, Ct values of 23.9 in healthy homozygotes and 26.3/27.2 in heterozygotes for G20210A were revealed. No homozygous subjects for G20210A and no double heterozygotes (for Leiden and G20210A mutations) were found. Despite significant differences in Ct values between the whole blood samples and the appropriate DNA extracts ($P<0.001$ for all the genotypes), their values lied inside the working range of the used method (0.01-35 ng/µl), (Fig. 2).

Comparing the genotypes determined in the extracts with those found in the modified whole blood, we manifest a complete concordance in their distribution; 61 patients revealed wt/wt genotype (frequency 0.61), 36 patients were heterozygotes (0.36), and three (0.03) were homozygous carriers of Leiden mutation. Frequencies of wild-type and mutant variants in the *FV* gene were 0.79 and 0.21. In the case of G20210A mutation in the *FII* gene, the wt/wt genotype was found in 95 cases (0.95), whereas M/wt genotype was present in five cases only (0.05). Frequencies of wild-type and mutant variants in the *FII* gene were 0.97 and 0.03, respectively.

Figure 3 demonstrates the stability of DNA molecules in the whole blood samples during 28 days of deep freezing interrupted by four cycles of thawing. The average Ct values for wild-type *FV* allele ranged between 22.8 and 24.2. The data achieved high consistency giving a coefficient of variability lower than 5 %, which indicated a high degree of integrity and amplificability of DNA in the samples.

Blood is not generally suitable material for spectrophotometric determination of quantity of nucleic acids. In this regard, we estimated the number of integral DNA molecules in blood by using quantitative real-time PCR in the *POLR2A* gene. Thus, the DNA content was influenced neither by RNA transcripts, nor by small DNA fragments, nor by structures interfering in UV spectrum.

PCR reactions could be inhibited by several blood components like hemoglobin, hematin or immunoglobulin G. To reduce their inhibitory effects Geiger *et al.* diluted blood samples before real-time PCR to 2 % in nuclease-free water and the diluted blood samples were subsequently heated for 10 min at 80 °C to inactivate potentially present blood-borne pathogens [13]. In their paper, however, quantitative parameters, Ct values, thresholds, or amplification curves are lacking. The determination of genotypes was performed by end-point analysis evaluating the fluorescence recorded during PCR cycling.

In our protocol, the whole blood samples provided satisfactory amplificability when the following steps were performed prior to real-time PCR: i) 1:1 water dilution, ii) 3 min heating at 90 °C, and iii) short spinning to obtain the supernatant. No reaction was observed when either the dilution or the heating step was omitted. The 1:1 dilution rate showed the PCR efficiency with Ct values inside the working range of the used methods. For that, the observed genotypes in the modified whole blood samples fully corresponded with the results of the appropriate DNA extracts. A higher dilution rate provided less reliable laboratory data.

Several papers focusing on various clinical applications of PCR previously used heating and dilution of blood samples before the amplification step [16-18]. Our study dealing with thrombophilic mutations for the first time demonstrated the possibility to compare PCR amplification of the whole blood samples to the extracts *via* Ct values and appropriate DNA concentrations.

We found the DNA median value of 18 ng/µl in the extracts and 0.2 ng/µl in the patients' whole blood, respectively. According to the theory of real-time PCR,

Table 1. Leiden mutation analysis, cycles of threshold determined in DNA extracts.

Genotype	DNA Extract		Whole Blood	
	wild-type allele	*mutant allele*	*wild-type allele*	*mutant allele*
wt/wt	18.5 (16.8-20.4)	n.d.	25.3* (21.0-28.7)	n.d.
M/wt	19.4 (18.1-20.8)	22.0 (20.7-23.7)	24.8* (20.4-29.1)	27.2* (23.1-31.9)
M/M	n.d.	20.9 (16.8-20.4)	n.d.	26.9* (24.8-29.2)

Abbreviations: n.d., not determined; * P<0.001 using Wilcoxon rank-sum test.

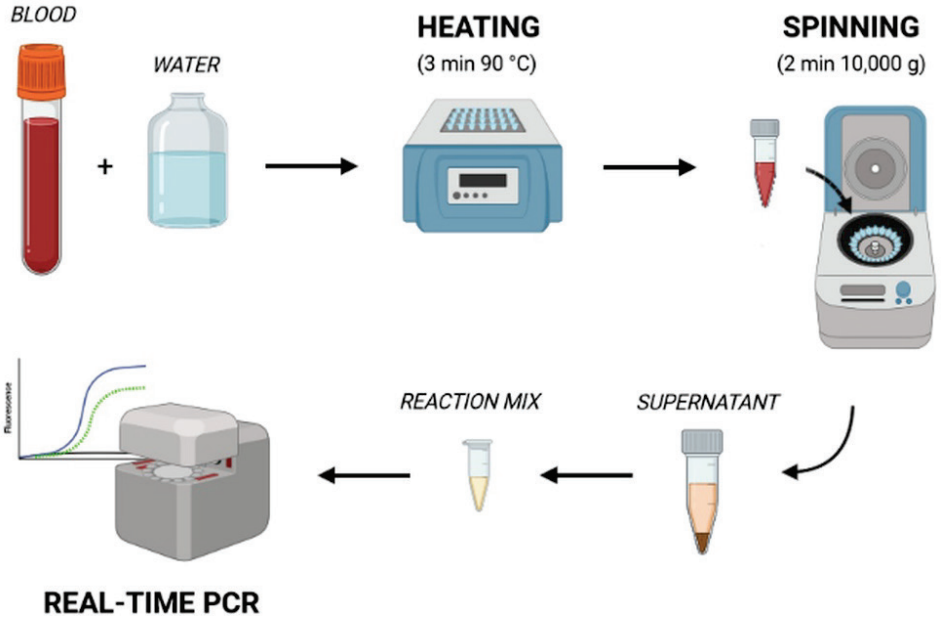

Fig. 1. Scheme of laboratory testing workflow. Created with BioRender.com.

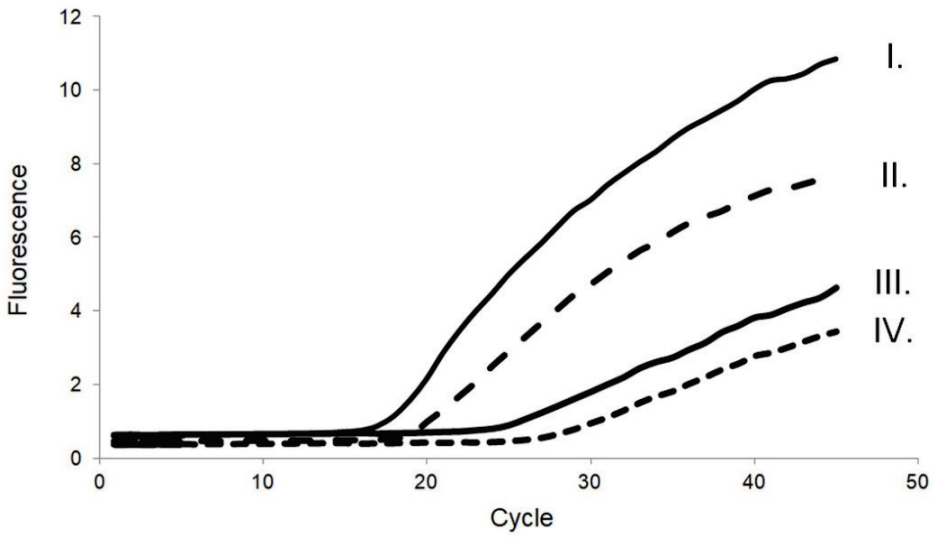

Fig. 2. Lines I and II show amplification curves for wild-type (solid line) and mutant (broken line) alleles in a DNA extract from a heterozygous subject carrying Leiden mutation. Line III and IV demonstrate amplificability of DNA present in a modified blood sample of the same subject.

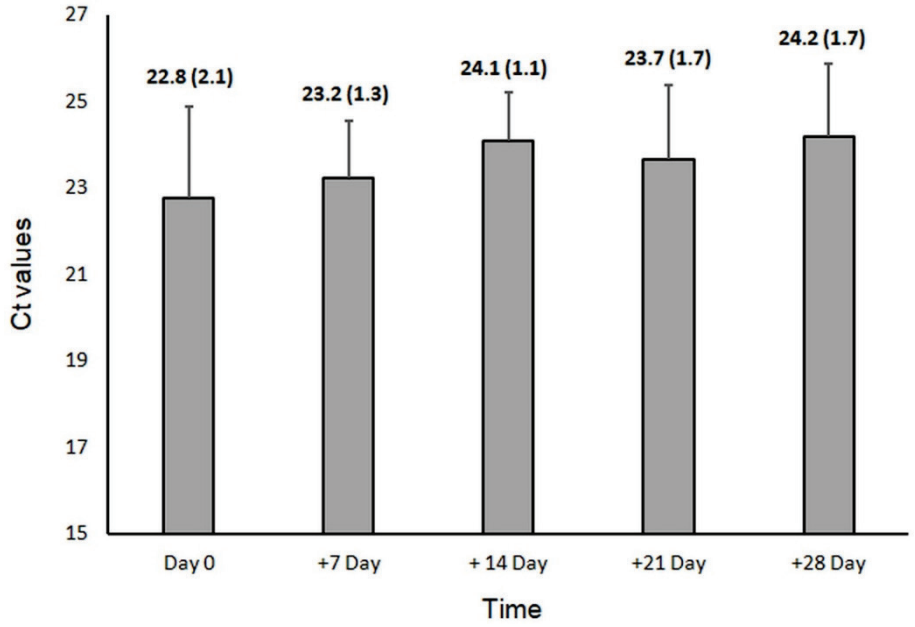

Fig. 3. Stability of DNA molecules in whole blood during 28 days freezing interrupted by four cycles of thawing.

a difference of 4-6 Ct values for the extracts and the blood samples could be predicted. Experimental data involving the samples of all three *FV* Leiden genotypes (wt/wt, M/wt, M/M) showed an average Ct difference of 5.7. This difference reflected unequal amounts of input DNA in PCR mixtures and seemed to be minimally influenced by the month storage of whole blood samples at -70 °C with repeated freezing-thawing. In G20210A mutation, an average Ct difference was 4.4. The discrepancies between the wild-type and the mutant alleles of the *FV* and *FII* genes, also apparent from Table 1 and Figure 2, were caused by the composition of reagents in the used commercial assays. Similar discrepancies were also seen in the heterozygous control samples contained in the kits.

LAMP is an isothermal amplification technique enabling rapid and sensitive detection of nucleic acids without standard thermal cycling. Several studies previously demonstrated a possibility to perform the testing without any whole blood modification, including analysis of thrombophilic mutations [14,19]. However, other published LAMP studies preferred sample dilutions and thermal pretreatment similar to our protocol [20]. Thus, LAMP and real-time technologies seem to require comparable amplification conditions for whole blood samples, including turnaround times (60-90 min), input sample volume, analytical sensitivity, and testing capacity.

Our study was performed on the background of COVID-19 pandemic in the world. Most automatic extraction systems in small and mid-size labs were centralized and re-configured to extract SARS-CoV-2 virus from nasopharyngeal swabs for real-time PCR testing. Under these laboratory conditions, using whole blood samples enabled us to perform a faster molecular analysis of thrombophilic mutations in patients of intensive care units.

In this study we used a non-random selection of the samples with enriched numbers of patients with Leiden mutation to obtain statistically valid laboratory data. We did not test any homozygous subjects for G20210A mutation because of their low population prevalence. A prior genotyping performed in 1527 healthy Czech individuals showed a population frequency of Leiden mutation of 4.5 % and 1.3 % for G20210A mutation [21]. We reported the results of genotyping more than two thousand Czech patients in risk of inherited thrombophilia, where the frequency of Leiden mutation of 14 % was estimated [22]. Further clinical effort is needed to elucidate the role of the thrombophilic mutations in the pathophysiology of COVID-19 infection.

The results of our study are limited by the fact that we tested EDTA blood samples only. Heparin salts were previously reported to decrease the activity of *Taq* polymerase in PCR reactions [23]. Several ways how to improve PCR amplification in heparinized blood were suggested: i) using a specific type of *Taq* polymerase, ii) addition of heparinase, lithium chloride or albumin into the mixture, or iii) dilution of DNA samples [23-26].

Heparin is, however, also present in blood

samples when administrated into the circulation as an anticoagulant drug. In therapy or prophylaxis of venous thromboembolism, pulmonary embolism, atrial fibrillation with embolization, acute and chronic consumptive coagulopathies, peripheral arterial embolism and other indications, heparin plasma levels are usually lower than 2 IU/ml. For comparison, optimized concentrations of unfractionated sodium, lithium and ammonium salts of heparin in collection tubes of various manufacturers range between 12-30 IU per 1 ml of blood. Thus, an inhibition effect of the heparin treatment is minute and it could be further minimized by blood collection into the tubes with EDTA or citrate salts. Significant inhibitory effects of drugs on PCR amplification were reported in cases of acyclovir, cisplatin, anthracyclines, bleomycin, D-cycloserine and mitomycin C [27,28].

In summary, we presented a new protocol for the detection of thrombophilic mutations *via* real-time PCR on the modified whole blood of COVID-19 patients. The blood modification is reliable, easy, cheap, and saving costs and turnaround time of the whole laboratory process. Its larger clinical application is only possible when a complete validation protocol and a subsequent accreditation procedure are successfully managed.

Conflict of Interest

There is no conflict of interest.

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