Technical Notes

No Inhibitory Effect of Heparinized Blood on Real-Time PCR Analysis of Thrombophilic Mutations

(real-time PCR / anticoagulants / whole blood / thrombophilic mutations / inhibition / cycle threshold)

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Abstract. We compared the efficiency of real-time PCR analysis of *FII* (c.*97G>A, G20210A) and *FV* Leiden (c.1601G>A) thrombophilic mutations in the samples obtained from venous blood treated with various anticoagulant agents (EDTA, heparin, and sodium fluoride with potassium oxalate), or from clotted venous blood; one hundred samples of wild-type subjects were tested. Genomic DNA extracts and whole blood specimens modified by 90 °C heating were analysed by real-time PCR analysis; cycle threshold values were subsequently evaluated. Real-time PCR analysis for the *FII* gene assay performed in DNA extracts from EDTA blood samples revealed a median Ct value of 19.3. Similar Ct values were apparent in the DNA extracts obtained from the heparinized blood and sodium fluoride with potassium oxalate-treated samples: 18.5 and 18.9, respectively. Significantly higher Ct values were found in extracts from clotted blood with medians of 20.6 (tubes with inert separation gel) and 20.5 (tubes without the gel, both $P < 0.001$). The data on the *FV* real-time PCR analysis were very comparable to the *FII* assay. In the modified whole blood, the samples treated with heparin salts showed significantly lower Ct values ($P < 0.001$) in both assays when compared with the samples with EDTA, sodium fluoride with potassium oxalate, and with the samples with clotted blood. Our results indicate that real-time PCR analyses of thrombophilic mutations were not negatively influenced by the presence of heparin salts in collection tubes. Blood samples with various anticoagulants might be exchangeable for each other when DNA analysis of thrombophilic mutations is required.

Introduction

Contemporary laboratory medicine harmonizes pre-analytical quality indicators and diagnostic procedures to provide equivalent and clinically acceptable data (Plebani et al., 2014; Plebani, 2018). An increasing number of validated *in vitro* diagnostic kits have improved inter-laboratory comparability in molecular biology techniques (Kaul et al., 2017; Kim et al., 2018). However, many pre-analytical factors such as types of biological material, fragmentation and chemical modification of nucleic acids still influence results of molecular analyses. The efficiency of extraction procedures and subsequent polymerase chain reaction (PCR) amplification are decreased by the presence of organic compounds such as haemoglobin, lactoferrin, immunoglobulin G, urea, melanin, bile salts, or salts of heparin, a potent anticoagulant agent (Gustafson et al., 1987; Holodniy et al., 1991). Beutler et al. (1990) stated that boiling DNA along with filtration on Sephadex G-75, altering pH followed by gel filtration, repeated ethanol precipitation, or titration with protamine sulphate did not allow successful PCR from heparinized blood specimens.

The proper mechanisms how heparin affects stability and integrity of DNA, and the course of standard PCR

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Abbreviations: Ct – cycle threshold, EDTA – ethylenediamine tetraacetic acid, PCR – polymerase chain reaction.

reactions are still unknown. It is possible that amplification of shorter products from heparinized blood DNA might improve the efficiency of the PCR performed. The technology of real-time PCR is based on formation and detection of short amplicons with a length of approximately 100 bp. This technique has a wide usage in various branches of clinical medicine including determination of *FII* (NM_000506.5:c.*97G>A, G20210A, rs1799963) and *FV* Leiden (NM_000130.4:c.1601G>A, rs6025) thrombophilic mutations.

In the current study, we compared the efficiency of real-time PCR analysis of thrombophilic mutations in the samples obtained from venous blood treated with various anticoagulant agents (EDTA, heparin, and sodium fluoride with potassium oxalate). The results of the study could possibly shorten the laboratory workflow in cases when blood samples are submitted in collection tubes with inappropriate anticoagulant agents, or clotted blood is drawn for molecular analysis.

Material and Methods

Subjects

The experimental group consisted of one hundred outpatients (56 females and 44 males with a median age of 66 years; range 22–96 years) of the University Hospital in Hradec Králové, Czech Republic. To obtain statistically valid data, the patients were characterized as wild-type individuals for both thrombophilic mutations during regular follow-ups and none of them used therapeutic anticoagulation. All the 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 FN HK, reference number 202209 P07, date 22 September 2022).

Blood collection

Non-clotted blood from each subject was collected into tubes containing tripotassium ethylenediamine tetraacetic acid (EDTA), lithium heparin, or sodium fluoride with potassium oxalate; clotted blood was collected into tubes with or without inert separation gel (all tubes BD Vacutainer system, Becton Dickinson, Franklin Lakes, NJ). Samples were stored at −80 °C until analysis; repeated thawing and freezing was avoided.

DNA extraction and blood modification

Genomic DNA was isolated from 200 µl of blood specimens by a MagCore Genomic DNA Whole Blood Kit (RBC Bioscience, New Taipei City, Taiwan) according to the manufacturer's instructions. The final elution volume was 200 µl. Apart from this standard extraction protocol, 20 µl of each blood sample was diluted with 20 µl of deionised water and incubated at 90 °C for 3 min. After that, the samples were spun at 10,000 *g* for 2 min and the resulting supernatant was immediately used for real-time PCR analysis.

Real-time PCR analysis

The mutations were determined by a gb HEMO FII (G20210A) Kit and gb HEMO FV Kit (both Generi Biotech, Hradec Králové, 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 work on the principle of allelic discrimination performed by real-time PCR with fluorescent hydrolytic probes in Rotor-Gene 6000 (Corbett Research, Sydney, Australia). For analysis, 4 µl of each DNA extract or 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. The threshold was set to 0.025. The verified working range of these assays corresponded with the Ct values determined in standard DNA extracts and lie between 15 and 30.

Statistics

All the experiments were done in duplicate, and the mean Ct value for each sample was calculated. Based on the Shapiro-Wilk test for normal data distribution, a parametric or nonparametric test was used to ensure the test sensitivity. The differences between the experimental and control subgroups were assessed using the Student's *t*-test or the Wilcoxon rank-sum test. The P values < 0.05 were considered statistically significant.

Results and Discussion

All the tested samples provided reliable amplification curves for wild-type (*wt*) alleles in *FII* and *FV* genes, and *wt*/*wt* homozygosity was determined in them. The Ct values lie inside the verified working range of both methods. Real-time PCR analysis for the *FII* gene assay performed in DNA extracts from EDTA blood samples revealed a median Ct value of 19.3 (range 18.4–20.2), see Table 1. Similar Ct values were shown in DNA extracts obtained from heparinized blood and sodium fluoride with potassium oxalate-treated samples: 18.5 (16.7–20.7) and 18.9 (16.0–20.0), respectively. Significantly higher Ct values than in the EDTA extracts were found in extracts from clotted blood with (median, 20.6; 18.5–27.3, $P < 0.001$) or without inert separation gel (20.5; 18.7–26.6, $P < 0.001$) in the tubes.

The data on the *FV* real-time PCR analysis were very comparable to the *FII* assay above. In the *FV* assay, the amplifiability and the Ct values in the DNA extracts from the clotted blood with or without the gel (20.6; 18.1–28.3 and 20.5; 18.5–27.4, both $P < 0.001$) significantly differed from those originated from the EDTA blood, heparinized blood and sodium fluoride with potassium oxalate-treated blood (medians, 18.7, 18.0 and 18.5, respectively), see Table 1.

As it is also apparent from Table 1, the real-time PCR analyses for *FII* and *FV* genes carried out in the modi-

Table 1. Thrombophilic mutations and cycle thresholds determined in the DNA extracts and whole blood samples

Sample	*FII*		*FV*	
	DNA extract	Whole blood	DNA extract	Whole blood
EDTA blood	19.3[ref] (18.4–20.2)	25.3[*] (22.6–27.7)	18.7[ref] (18.0–19.6)	26.2[*] (24.1–27.8)
Heparinized blood	18.5 (16.7–20.7)	19.8[#] (17.7–25.4)	18.0 (16.0–20.6)	20.5[*#] (17.9–23.6)
NaF/oxalate blood	18.9 (16.0–20.0)	24.4[*] (20.1–26.4)	18.5 (16.0–19.8)	24.6[*] (21.9–28.7)
Clotted blood	20.5[*] (18.7–26.6)	25.6[*] (22.3–29.5)	20.5[*] (18.5–27.4)	26.4[*] (22.5–29.2)
Clotted blood with gel	20.6[*] (18.5–27.3)	25.8[*] (22.3–30.4)	20.6[*] (18.1–28.3)	25.7[*] (21.9–30.1)

[ref]reference group for the assay, [*]P < 0.001 to the reference group, [#]P < 0.001 to EDTA whole blood samples using Wilcoxon rank-sum test

fied whole blood showed Ct values higher by 5–6 cycles in the samples with EDTA, sodium fluoride with potassium oxalate, and in the case of both types of clotted blood. In contrast, the presence of heparin in the collection tubes significantly decreased the Ct values in this type of modified whole blood samples (*FII*, 19.8, 17.7–25.4, P < 0.001 and *FV*, 20.5, 17.9– 23.6, P < 0.001) and improved PCR amplifiability in the reaction tubes (Fig. 1).

Using collection tubes with a proper anticoagulant agent belongs to fundamental pre-analytical factors for obtaining reliable laboratory data. EDTA salts are recommended for routine haematological testing, heparin salts are preferred in clinical biochemistry, sodium citrate is suitable for coagulation studies, and sodium fluoride with oxalate salts are commonly used in measurement of fasting plasma glucose. The influence of various

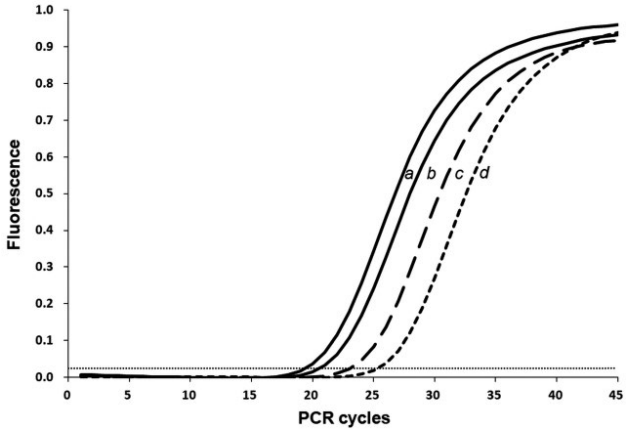

Fig. 1. Typical real-time PCR amplification curves (*FII* c.*97G>A analysis) for DNA extracted from EDTA blood (line a), whole blood with heparin (line b), whole blood with EDTA (line c), and whole blood with sodium fluoride and potassium oxalate (line d).

anticoagulant agents on the results of biochemical analyses in medical and veterinary practice has been previously published (Imafuku et al., 2002; Sacchetto et al., 2014). Their chemical or physical interference is particularly apparent in collection tubes with an insufficient blood volume drawn. EDTA and citrate salts are considered the best anticoagulants to extract high molecular weight DNA. As they have very similar effects on PCR analysis (Gustafson et al., 1987) and collection tubes with EDTA are used much more frequently, the inhibitory effects of citrate salts were not examined in this study.

Heparin was many times reported to inhibit a conventional PCR reaction (Beutler et al., 1990; Holodniy et al., 1991; Izraeli et al., 1991; García et al., 2002). During the extraction process, heparin salts are co-extracted with DNA molecules from cells, and subsequently they can interfere with PCR, probably by conjugation with the *Taq* DNA polymerase peptide chain (Yokota et al., 1999). The inhibitory effects of heparin depend on magnesium or manganese ions, DNA dilution, type of DNA polymerase and its amount in a collection tube (Cai et al., 2018).

Optimized concentrations of unfractionated salts of heparin in collection tubes of various manufacturers range between 12–30 units per 1 ml of blood. 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 10,000 units (blood concentrations are < 2 units/ml); however, during cardiopulmonary bypass, heparin concentrations can reach 50,000 units; blood concentrations are approximately 10 units/ml (Kondratov et al., 2016).

To the best of our knowledge, no study evaluating the influence of anticoagulant agents on real-time PCR analysis of thrombophilic mutations has been published yet. Our data demonstrate that the collection tubes containing sodium fluoride and potassium oxalate achieved

similar efficiency of real-time PCR as those with EDTA, in both thrombophilic assays and regardless of whether DNA extracts or modified whole blood was used as a template.

Furthermore, the extracts prepared from the heparinized blood provided concordant Ct values for both assays with either EDTA or sodium fluoride with potassium oxalate-treated samples. Thus, in contrast with the studies above that used the conventional PCR, our data showed no inhibition effect of heparin on real-time PCR reactions. The found discrepancy has at least two possible explanations. The first is that real-time PCR protocols generate very short amplicons with a weaker inhibitory effect of heparin when compared to the conventional PCR. Alinezhad et al. (2022) recently documented that heparin inhibited a real-time PCR reaction in which a 681 bp amplicon of CPV virus was formed. This inhibitory effect was eliminated by heating the viral samples or DNA extracts at 65/85 °C for 2 hours (Alinezhad et al., 2022). The second explanation is that our fully automated extraction procedure minimized the inhibitory effects of heparin. Ding et al. (2011) reported that DNA extracts obtained from heparinized blood by the EasyMag extraction machine showed no inhibitory effect of heparin on real-time PCR analysis of CMV virus.

To find answers to the question why heparin did not interfere with real-time PCR analysis of thrombophilic mutations, we performed both real-time PCR reactions directly from whole blood samples slightly modified by 3 min heating at 90 °C and spinning. Free heparin and other anticoagulants had remained in the supernatant, which was immediately added into the PCR reaction tube. The difference of 5–6 Ct values between the extracts and whole blood samples observed in EDTA and sodium fluoride with potassium oxalate salts reflects unequal amounts of input template molecules added to the PCR mixtures (Beránek et al., 2022).

Similarly as in the DNA extracts, the presence of heparin in the whole blood samples did not influence the efficiency of real-time PCR in the *FII* and *FV* assays. Even the Ct values for heparinized blood were significantly lower than those for EDTA or sodium fluoride with potassium oxalate blood in both assays and comparable with the results of analysis performed with standard DNA extracts (Table 1). In our protocol, we heated the blood samples at 90 °C for 3 minutes before spinning and obtaining the supernatant. In agreement with our results, Alinezhad et al. (2022) showed that heparin residues had lesser interference than EDTA in PCR reactions after two-hour heating.

The presented data indicate that not only the used extraction procedure, but also heparin itself influences the course of amplification curves for the thrombophilic mutations. It is not completely clear why heparin-treated blood and/or its supernatant improve the efficiency of real-time PCR in comparison to blood samples containing other anticoagulants. Several explanations of these findings could be possible: i) low numbers of template DNA molecules in the whole blood supernatants are diluted in PCR mixtures, which decreases the probability of heparin inhibitory effects; ii) inhibitory effects of EDTA and sodium fluoride with potassium oxalate are not minimized in whole blood, and therefore these effects manifest themselves more strongly in the supernatants after heating; iii) heparin, a polysaccharide, enables more efficient release of DNA molecules in whole blood samples; iv) inhibitory effects of heparin on DNA polymerase are mainly apparent in cases when the process of reverse transcription is carried out before PCR.

Heparin was previously observed to inhibit catalytic activities of several enzymes. Apart from DNA polymerase mentioned above, affected activities were also shown in ribonucleases and reverse transcriptases (Wang et al., 1992). In concordance with these findings, several papers documented inhibitive effects of heparin on gene expression studied *via* reverse transcription and PCR (Holodnyi et al., 1991; Izraeli et al., 1991; Wang et al., 1992, Bai et al., 2000; Ding et al., 2011; Li et al., 2016). As standard RNA extraction procedures do not remove heparin salts from blood samples, plasma, urine and perfusate, various approaches to minimizing heparin effects on mRNA or miRNA analysis were reported, i.e., sample dilution, heating, sonication, adding heparinase, nitrous acid, ascorbic acid or hydrogen peroxide (Beutler et al., 1990; Kondratov et al., 2016, Li et al., 2016; Roest et al., 2021).

In the last part of this study, we investigated PCR amplifiability of DNA from clotted blood in collection tubes with or without inert separation gel in comparison with EDTA blood samples. Despite the fact that any biochemical or genetic analysis with fully coagulated blood is difficult, in our hands the DNA extracts and modified whole blood samples showed satisfactory Ct values and 100 % concordant genotyping results of analysis of the thrombophilic mutations to the EDTA samples.

Summarizing our findings, none of the performed real-time PCR analyses of thrombophilic mutations was negatively influenced by the presence of heparin in standard concentrations in the collection tubes. It seems that real-time PCR analysis generating products of approximately 100 bp amplicons is not inhibited by heparin salts. Thus, blood samples collected in EDTA, sodium fluoride with potassium oxalate, and heparin might be exchangeable for each other when DNA analysis of thrombophilic mutations is required. This fact could shorten the current laboratory workflow and decrease the number of reasons for sample refusal. Further studies shall be needed to extend our findings in other real-time PCR assays used in clinical medicine.

Conflict of interest

There is no conflict of interest.

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