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Direct blood PCR: TaqMan-probe based detection of the venous thromboembolism associated mutations factor V Leiden and prothrombin c.20210G > A without DNA extraction

Kathrin Geiger[a], Andreas Leiherer[a,b,c], Eva-Maria Brandtner[a], Peter Fraunberger[b], Heinz Drexel[a,c,d,e], Axel Muendlein[a,*]

[a] Vorarlberg Institute for Vascular Investigation and Treatment (VIVIT), Feldkirch, Austria
[b] Medical Central Laboratories, Feldkirch, Austria
[c] Private University of the Principality of Liechtenstein, Triesen, Liechtenstein
[d] Division of Angiology, Swiss Cardiovascular Center, University Hospital of Berne, Berne, Switzerland
[e] Drexel University College of Medicine, Philadelphia, PA, USA

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ABSTRACT

Background: Practically, the initial step of genetic analysis is the extraction of DNA from blood or other cells, which is often time consuming and cost-intensive. We aimed at establishing a real-time PCR protocol for the detection of the venous thromboembolism associated mutations factor V Leiden (F5 c.1691G > A; p.R506Q) and prothrombin (F2) c.20210G > A from whole blood, without DNA extraction.

Methods: F5 c.1691G > A (p.R506Q) and F2 c.20210G > A mutations were determined in 205 EDTA anti-coagulated whole blood samples from patients who underwent routine clinical genotyping using the DirectBlood Genotyping PCR Kit (myPOLS Biotec, Konstanz, Germany) together with in-house developed TaqMan primer-probe assays.

Results: Validity score values of genotype calls using whole blood were similar and did not significantly differ compared to those using genomic DNA as substrate in PCR. Mutation analysis of 205 whole blood samples showed a negligible PCR dropout rate (one in 410 reactions) and were in 100% concordance with results obtained by conventional genotyping.

Conclusion: We successfully established a robust and valid real-time PCR protocol for the detection of the venous thromboembolism associated mutations F5 c.1691G > A (p.R506Q) and F2 c.20210G > A directly from whole blood.

1. Introduction

Factor V Leiden (F5 c.1691G > A; p.R506Q; rs6025) [1] and the prothrombin (F2) c.20210G > A (rs1799963) [2] mutation are the major inherited risk factors of venous thromboembolism manifested as deep venous thrombosis or pulmonary embolism and have been further associated with a higher risk of myocardial infarction [3], stroke [4], and miscarriage [5]. Consequently, routine genetic testing is recommended in several clinical circumstances, particularly in patients with venous thrombosis at younger age, a strong family history of thrombotic disease, or in women with a history of venous thrombosis during pregnancy [6].

A variety of methods have been proposed for the detection of these genetic variants [7,8]. Probably the most commonly used are real-time polymerase chain reaction (PCR) assays using TaqMan-probes due to their underlying relatively simple technique and excellent analytic validity [9–12]. TaqMan technology relies on the 5′-3′ exonuclease activity of the thermostable Taq-DNA polymerase in combination with allele-specific probes labeled with a distinct fluorescent reporter dye on the 5′-end and a quencher on the 3′-end [13]. An allele-specific fluorescence signal is generated by cleavage and separation of the reporter dye from the quencher when hybridization of the probe to the complementary allelic sequence occurs during PCR. Incorporation of locked nucleic acids (LNAs) into the

Abbreviations: PCR, polymerase chain reaction; LNAs, locked nucleic acids; 6FAM, 6-carboxy-fluorescein; HEX, hexachloro-fluorescein; BHQ1, Black Hole Quencher™ 1

* Corresponding author.
E-mail address: axel.muendlein@vivit.at (A. Muendlein).

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probe sequence [14] or 3′-end attachment of a minor groove binder molecule [15] enhances binding affinity to complementary sequences and allows the generation of relatively short yet highly allele-specific probes.

However, apart from the fast and accurate TaqMan-probe based allele-detection technique, the initial step of genetic analysis practically is the extraction of DNA from blood or other cells, which is often time consuming and cost-intensive. Direct PCR analysis using blood as substrate, without prior DNA extraction and purification, is generally hampered by PCR inhibition induced by blood components, especially hemoglobin and immunoglobulin G [16]. To overcome this problem, scientists have tried to develop robust DNA polymerases and to identify PCR additives that may allow PCR amplification in the presence of blood compounds [16–20]. However, despite these technological real-time PCR using whole blood directly as a DNA source remained inferior [16], particularly in terms of efficiency. No real-time PCR protocol for the detection of the F5 c.1691G > A (p.R506Q) and F2 c.20210G > A mutations directly from blood has been reported in the current literature, to date.

In the present study we, therefore, investigated the robustness and analytic validity of a recently launched Taq-polymerase kit specifically adapted for real-time PCR directly from blood, the DirectBlood Genotyping PCR Kit (myPOLS Biotec, Konstanz, Germany), together with in-house TaqMan primer-probe assays designed for the detection of the genetic variants F5 c.1691G > A (p.R506Q) and F2 c.20210G > A. We evaluated i) the impact of blood on the performance of the DirectBlood Genotyping PCR Kit and our assays using whole blood and extracted genomic DNA as substrates and ii) the reliability of our direct blood PCR protocol by re-genotyping over 200 blood samples from patients who previously underwent routine F5 c.1691G > A (p.R506Q) and F2 c.20210G > A genotyping.

2. Methods

2.1. Patients' samples

In the present study 205 EDTA anti-coagulated whole blood samples from patients who underwent routine clinical F5 c.1691G > A (p.R506Q) and F2 c.20210G > A genotyping between 2016 until present were used. Additionally, 25 archived genomic DNA samples for each mutation were recovered to evaluate the performance of our assays using different substrates. Blood samples as well as DNA samples were stored at −20 °C after initial routine genotyping. Samples were selected based on previously determined genotypes to increase the number of risk-allele-carriers compared to naturally occurring genotype frequencies. With respect to the F5 c.1691G > A (p.R506Q) mutation, the present study included 104 carriers homozygous for the common allele, 100 carriers of the heterozygous genotype, and one carrier homozygous for the rare allele. With respect to the F2 20210G > A genetic variant, the study included 174 carriers homozygous for the common allele, 30 carriers of the heterozygous genotype, and one carrier homozygous for the rare allele. All patients gave written confirmed consent for clinical F5 c.1691G > A (p.R506Q) and F2 c.20210G > A testing.

2.2. Routine genotyping

For routine genotyping, genomic DNA was extracted from 200 µL EDTA blood using the peqGOLD® Blood DNA Mini kit (VWR, Vienna, Austria) and eluated in 200 µL elution buffer corresponding to a DNA concentration of up to 75 ng/µL according to the information of the manufacturer. Genotyping of the F5 c.1691G > A (p.R506Q) and F2 c.20210G > A variants was carried out by commercially available TaqMan® genotyping assays (assay ID: C_11975250_10 and C__8726802_20, respectively; Thermo Fisher Scientific, Waltham, MA, USA) used with TaqMan® Genotyping PCR Master Mix (Thermo Fisher Scientific) on a LightCycler® 480 Real-Time PCR System (F. Hoffmann-La Roche Ltd., Basle, Switzerland).

Table 1
Primer and probe sequences for F5 c.1691G > A (p.R506Q) and F2 20210G > A detection.

F5 c.1691G > A (p.R506Q)	
F5 forward	5′ GAA AGG TTA CTT CAA GGA CAA AAT ACC
F5 reverse	5′ AGA CAT CGC CTC TGG GCT AAT AG
G1681 (*R*506)-probe	6FAM - ACA GGc gAg GAA T - BHQ1
A1681 (Q506)-probe	HEX - ACA GGc aag GAA T - BHQ1
F2 c.20210G > A	
F2 forward	5′ GCT CCT GGA ACC AAT CCC GTG
F2 reverse	5′ CCA GAG AGC TGC CCA TGA ATA G
G20210-probe	6FAM - CTC AGc gAg CCT C - BHQ1
A20210- probe	HEX - CTC AGc aag CCT C - BHQ1

Locked nucleotides (LNA) are denoted in lower case; the nucleotide complementary to the target mutation is underlined. 6FAM, 6-carboxy-fluorescein; HEX, hexachloro-fluorescein; BHQ1, Black Hole Quencher 1™.

2.3. Direct blood TaqMan-PCR

2.3.1. Primers and probes

TaqMan probe based genotyping assays were designed to detect the F5 c.1691G > A (p.R506Q) and F2 20210G > A mutations. Each genotyping assay includes a pair of PCR primers flanking the respective genetic variation and two allele-specific TaqMan probes containing LNAs and distinct fluorescent reporter dyes to detect the specific mutation. TaqMan probes labeled with the 5′ fluorescent reporter dye 6-carboxy-fluorescein (6FAM) are specific for the respective common alleles and TaqMan probes labeled with hexachloro-fluorescein (HEX) are specific for the respective rare alleles. Adhering to recommended fluorescent reporter dyes and non-fluorescent quencher combinations, a non-fluorescent Black Hole Quencher™ 1 (BHQ1) is attached to the 3′ end of each TaqMan probe [14]. Primer and probe sequences are given in Table 1. All oligonucleotides were synthesized by Microsynth Inc. (Balgach, Switzerland).

2.3.2. Sample preparation

Blood samples were thawed at room temperature and homogenized by vortexing. Blood samples were diluted to 2% in nuclease-free water by adding of 10 µL blood to 490 µL water. Diluted blood samples were heated for 10 min at 80 °C to inactivate potentially present blood-borne pathogens. After the heating step, blood samples were placed on ice and immediately used as substrates in PCR.

2.3.3. TaqMan PCR

The DirectBlood Genotyping PCR Kit (myPOLS) was delivered as a freeze-dried lyocake together with a respective rehydration buffer. According to the instructions of the manufacturer, the lyocake was rehydrated by adding 218 µL of the rehydration buffer, resulting in 250 µL of a ready-to-use 2× PCR master mix. Synthesized primer and probes were used to produce 4× genotyping assays with concentrations of 3600 nM of each PCR primer and 800 nM of each TaqMan probe.

PCR was performed in a 20 µL volume containing 10 µL of 2× DirectBlood Genotyping PCR master mix, 5 µL of the in-house manufactured 4× primer-probe assays, and 5 µL of 2% diluted blood. Alternatively, 5 µL of previously extracted, undiluted genomic DNA was used to compare the performance of the DirectBlood Genotyping PCR Kit using different substrates. All TaqMan PCRs were carried out on a LightCycler® 480 Real-Time PCR System (Roche Diagnostics, Vienna, Austria) under the following thermocycling conditions: 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Non-template controls as well as samples homozygous for the respective rare allele were included in each run as duplicates.

Genotypes were determined by LightCycler® software 1.5. Genotyping analysis software groups similar samples and automatically calls genotypes. Additionally, the software assigns a score between 0 and 1 to each call, indicating the validity of the call (0 = invalid; 1 = highly valid). Samples failed to be automatically called were manually assigned to the most probably genotype by visual inspection or determined as uncertain or negative.

2.4. Statistics

The Kolmogorov-Smirnov test was used as a test for normality of score values obtained by LightCycler® software 1.5 indicating that score values were not normally distributed. Differences in score values using either whole blood or genomic DNA as substrates were tested for statistical significance with the non-parametric Wilcoxon test. *P*-values < .05 were considered significant. Statistical analyses were performed with SPSS 25.0 for Windows (IBM, Armonk, New York, USA).

3. Results

The impact of whole blood on the performance of the DirectBlood Genotyping PCR Kit (myPOLS) together with in-house TaqMan primer-probe assays was evaluated by using whole blood samples and corresponding genomic DNA samples of the same patients as substrates. Scatter plots obtained by real-time PCR of the F5 c.1691G > A (p.R506Q) and F2 c.20210G > A mutations using either whole blood or genomic DNA are displayed in Fig. 1. Overall, PCRs using undiluted genomic DNA samples as substrates showed stronger fluorescence signals compared to whole blood samples most probably reflecting different amounts of input DNA. However, all genotypes from direct blood PCR could be called automatically and were in 100% concordance with those using genomic DNA as substrate in PCR. Furthermore, validity score values of automatically called genotypes using whole blood were highly similar and did not differ significantly compared to those using genomic DNA [F5 c.1691G > A (p.R506Q): Median score value = 0.97

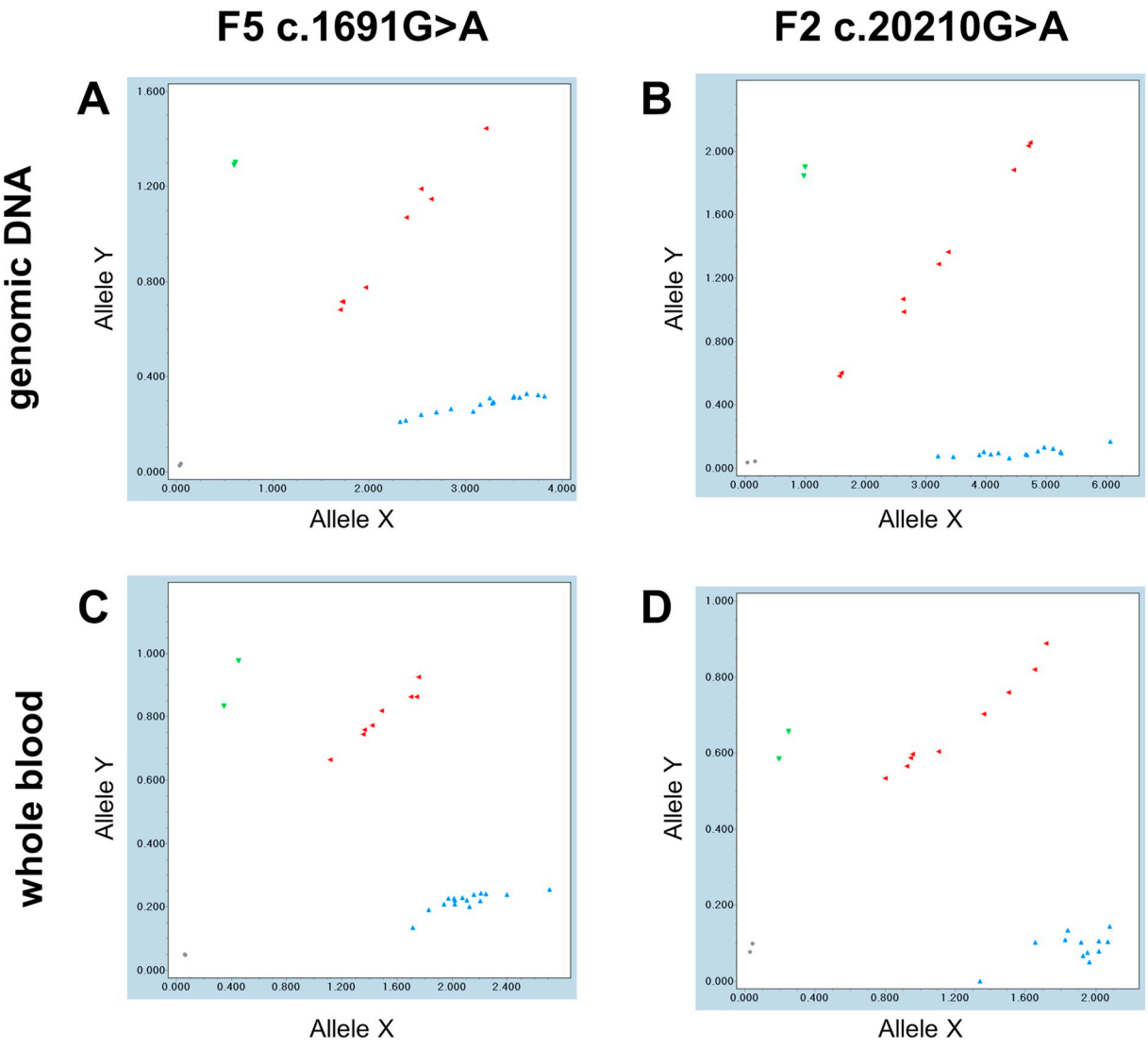

Fig. 1. Scatter plots obtained by real-time PCR of F5 c.1691G > A (p.R506Q) (A, C) and F2 c.20210G > A (B, D) mutations using either genomic DNA (A, B) or whole blood (C,D) as substrates. Allele X = common allele, allele Y = rare allele. Allele X homozygotes are clustered in blue; allele Y homozygotes are clustered in green; heterozygotes are clustered in red. Non-template controls (NTCs) are clustered in black. NTCs as well as samples homozygous of the respective rare allele were included in each run as duplicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(range = 0.90–1) and 0.98 (range = 0.90–1) for whole blood and for genomic DNA, respectively (p = .551); F2 c.20210G > A: Median score value = 0.94 (range = 0.85–1) and 0.94 (range = 0.88–1) for whole blood and for genomic DNA, respectively (p = .367)].

To evaluate the reliability of our direct blood PCR protocol, we compared the results previously obtained from conventional routine genotyping of the F5 c.1691G > A (p.R506Q) and F2 c.20210G > A variants with results now obtained from direct blood PCR totally including 205 subjects. Raw data including fluorescence signal strength and score values of each individual analysis are given in Supplementary Table S.1. For the F5 c.1691G > A (p.R506Q) mutation, genotypes of 203 samples were automatically called correctly (median score = 0.96; range = 0.68–1), one sample was automatically called as uncertain and one sample as negative. The genotype of the uncertain samples could be assigned correctly by manual editing after visual inspection (Supplementary Fig. S.1). The analysis of the negative sample was repeated showing the correct automatically called genotype (score = 0.94) in the repeated analysis pointing to handling errors in the initial analysis. With respect to the F2 c.20210G > A variant, genotypes of all 205 samples were automatically called correctly (median score = 0.97; range = 0.65–1).

4. Discussion

In the present study, we successfully developed a robust and valid real-time PCR protocol for the detection of the venous thromboembolism associated mutations F5 c.1691G > A (p.R506Q) and F2 c.20210G > A directly from blood: Mutation analysis of 205 whole blood samples showed a negligible PCR dropout rate (one in 410 initial reactions) and a 100% concordance with results previously obtained by conventional genotyping. The reported method is reliable, cost-effective, and accelerates time to result supporting rapid clarification of a potential underlying genetic background of venous thromboembolism.

Our protocol is based on the use of the commercially available DirectBlood Genotyping PCR Kit (myPOLS) together with in-house developed TaqMan-assays for real-time mutation detection. Of note, also other suppliers have released robust DNA polymerases resistant to inhibitors present in whole blood [21] such as the Phusion® Blood II DNA Polymerase (Thermo Fisher Scientific), the Hemo KlenTaq Polymerase (New England Biolabs, Ipswich, MA), the KAPA Blood DNA Polymerase (Kapa Biosystems, Wilmington, MA), or the KOD FX DNA Polymerase (Toyobo, Osaka, Japan). However, these polymerases show either proofreading 3′-5′ exonuclease activity or lack 5′-3′ exonuclease activity and, therefore, are not applicable for TaqMan-based real-time PCR. The DNA polymerase included in the DirectBlood Genotyping PCR Kit with its 5′-3′ exonuclease activity and its robustness makes the kit particularly suitable for TaqMan-based real-time PCR directly from whole blood samples.

The DirectBlood Genotyping PCR Kit is available as a freeze-dried lyophilisate and, therefore, can be shipped at ambient temperature without the need for cool packaging. The lyophilisate and its rehydration buffer may contain substances to preserve polymerase functionality during the freeze-drying process as well as to increase PCR inhibitor resistance. In this regard, the disaccharide trehalose is known to be both, a stabilizer of enzyme activity in dry conditions [22] and a potent PCR enhancer [23] and might, therefore, be a component of the DirectBlood Genotyping PCR Kit. Notably, trehalose or other PCR enhancers significantly lower DNA melting temperature [23] and consequently may affect probe specificity. Therefore, performance and specificity of primer-probe assays established under regular PCR conditions may have to be re-evaluated using the DirectBlood Genotyping PCR Kit. Particularly, the use of relatively short and highly allele-specific probes is required which in our study was achieved by incorporation of LNAs into TaqMan probe sequences [14]. Alternatively, attachment of a minor groove binding molecule at the 3′-end of the probes may probably show similarly marked effects [15,24]. However, minor groove binder probes are proprietary to Thermo Fisher Scientific whereas the main patent of LNAs expired in 2017 making LNA oligonucleotides ubiquitously available.

Blood components such as hemoglobin and hematin are known to significantly quench fluorescence signaling [16] limiting final blood concentration and consequently DNA input in TaqMan-probe based real-time PCR. In this regard, as stated in the manufacturers' instructions of the DirectBlood Genotyping PCR Kit the final concentration of whole blood should be 0.5%. According to the normal concentration of white blood cells varying between 4000 and 10,000 per microliter in whole blood of healthy adults [25], 5 µL of 2% diluted whole blood as used in PCR corresponds to 800–2000 genome equivalents or 2.9–7.4 ng genomic DNA, which appears sufficient for germline mutation detection by real-time PCR. However, sensitivity of TaqMan-based detection of blood-borne pathogens or somatic mutations from diluted blood samples may be limited especially in cases of low levels and low allelic burden, respectively. Moreover, a very low white blood cell count, which can be caused by certain autoimmune disorders, cancer, viral infections or medication intake [26–28], may also affect sensitivity of germline mutation detection by real-time PCR directly from diluted blood samples. That said, in our study we did not correct for white blood cell count and received valid results from all included patients.

In summary, we present a reliable protocol for the detection of F5 c.1691G > A (p.R506Q) and F2 c.20210G > A mutations directly from whole blood saving costs and time. The described method is easily reproducible in every laboratory with real-time PCR technology and may therefore stimulate further research and assist the clinician in the diagnosis of inherited venous thrombosis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2018.11.016.

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Conflicts of interest

The authors declare that they have no conflicts of interest related to the contents of this article.

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