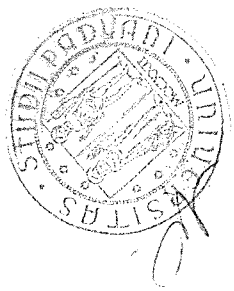
SELEZIONE PUBBLICA N. 2019S2, PER TITOLI ED ESAMI, PER LA STIPULA DI N. 1 CONTRATTO DI LAVORO A TERMINE, CATEGORIA D, POSIZIONE ECONOMICA D1, AREA TECNICA, TECNICO-SCIENTIFICA ED ELABORAZIONE DATI, TEMPO PIENO, PER 12 MESI, AI SENSI DEL D.LGS. 30.03.2001, N. 165 E SS.MM.II., DEL D.LGS. 15.06.2015, N. 81 IN QUANTO COMPATIBILE, E DEL C.C.N.L. DEL 19.04.2018, PRESSO IL DIPARTIMENTO DI MEDICINA – DIMED DELL'UNIVERSITÀ DEGLI STUDI DI PADOVA

9 aprile 2019

COLLOQUIO

1. Diagnosi differenziale dei difetti di proteina S: tecniche e classificazione



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9 aprile 2019

COLLOQUIO

2. I test di trombino generazione nei portatori di trombofilia ereditaria

SELEZIONE PUBBLICA N. 2019S2, PER TITOLI ED ESAMI, PER LA STIPULA DI N. 1 CONTRATTO DI LAVORO A TERMINE, CATEGORIA D, POSIZIONE ECONOMICA D1, AREA TECNICA, TECNICO-SCIENTIFICA ED ELABORAZIONE DATI, TEMPO PIENO, PER 12 MESI, AI SENSI DEL D.LGS. 30.03.2001, N. 165 E SS.MM.II., DEL D.LGS. 15.06.2015, N. 81 IN QUANTO COMPATIBILE, E DEL C.C.N.L. DEL 19.04.2018, PRESSO IL DIPARTIMENTO DI MEDICINA – DIMED DELL'UNIVERSITÀ DEGLI STUDI DI PADOVA

9 aprile 2019

COLLOQUIO

3. I difetti di fattore V della coagulazione

WHO/BS/04.1997
English Only

EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION
Geneva, 15 to 19 November 2004

A COLLABORATIVE STUDY ON THE PROPOSED 1ST INTERNATIONAL GENETIC REFERENCE PANEL FOR FACTOR V LEIDEN (G1691A), HUMAN gDNA

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SUMMARY

Forty-one laboratories participated in an international collaborative study to assess the suitability of a panel of three gDNA samples as the 1st International Genetic Reference Panel for factor V Leiden (FVL). The code numbers of the materials were 03/254 (FV wild type), 03/260 (FVL homozygote) & 03/248 (FVL heterozygote). The participants evaluated the panel against their in-house controls which were known patient samples and commercial controls. In total, 859 genotype tests were carried out on the panel, with an error rate of 0.7 %. The errors were not related to specific samples of the panel or to any specific techniques. The findings of this study have indicated that this panel is suitable to be used as a reference material for genotyping of factor V Leiden. It is therefore recommended that the three gDNA samples be established as the 1st International Genetic Reference Panel for factor V Leiden. Twenty-nine out of the 41 participating laboratories agreed with the recommendation. Twelve laboratories have not replied. This recommendation was also approved by the Scientific and Standardization Committee (SSC) of the ISTH (International Society on Thrombosis and Haemostasis) in June 2004.

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INTRODUCTION

The factor V Leiden polymorphism results in a slower inactivation rate of activated factor V by activated protein C[1] and it is associated with 5-10x increased risk of venous thrombosis in heterozygotes and a 50-80x increase in homozygotes[2-5]. The factor V gene is located on chromosome 1q23, spans more than 80kb and contains 25 exons.[6] The factor V gene defect occurs in exon 10 where there is a G-A substitution at nucleotide 1691.[7] The highest prevalence of the mutation has been found in European populations of Caucasian origin, most notably in Cypriot Greek (13%)[8], Swedish (11%)[9], French (10%)[10], British (9%)[8], German (9%)[11], and Dutch (5%)[12]. In contrast the mutation appears to be rare among Chinese [8,13-15] and absent in Japanese[16] and Africans (Negroid)[8, 17]. As a consequence of the high incidence of the mutation, testing for factor V Leiden is one of the most frequent genotyping tests performed in clinical laboratories. The frequency of testing is also likely to increase with organisations such as the WHO, funding programmes on association of pre-existing risk factors such as a previous history of thrombosis, use of oral contraceptives or hormone replacement therapy and genetic susceptibility with venous thrombosis and air travel[18]. External quality assurance schemes have shown that errors in genotyping on factor V Leiden do occur. These errors can have a significant and long-lasting impact on the patient, particularly because genotyping tests are usually carried out only once on any one patient. This is in contrast to many other pathology tests which are often carried out on different occasions, allowing errors to be identified.

Most laboratories use blood or extracted DNA samples from patients with the known polymorphism as their in-assay references. These reference materials are not widely available from commercial sources and laboratories that are new to the field rely on materials from genetic reference laboratories. However, a continual supply of a stable and reliable reference material for this polymorphism is not currently guaranteed. We have therefore produced a panel of gDNA materials. The gDNA was extracted from immortalised cell lines produced by Epstein-Barr virus (EBV) transformation of blood from donors who were known to carry the wild type, homozygote and heterozygote genotypes for factor V Leiden, with a view to establish these materials as the 1st International Genetic Reference Panel for Factor V Leiden. These materials are well characterised with confirmed genotypes from consenting donors and because they are obtained from cell lines future supplies of identical materials are ensured. They will be of paramount importance for validation of commercial *in vitro* diagnostic kits for factor V Leiden and for laboratories setting up new techniques and for validating existing techniques after a change of reagents, operator or equipment.

AIM OF STUDY

The main aim of the study was to evaluate a panel of 3 freeze-dried gDNA samples extracted from EBV transformed cell lines known to carry the genotypes for wild type factor V, homozygote factor V Leiden and heterozygote factor V Leiden in an international collaborative study involving laboratories using a wide variety of genotyping techniques, thereby assessing its suitability as the WHO 1st international genetic reference panel for Factor V Leiden.

CANDIDATE MATERIALS

Blood samples were collected from three consenting donors; each was tested and found negative for anti-HIV 1/2, HBsAg and anti-HCV. The genotypes of the donors were confirmed by RFLP (restriction fragment length polymorphism) using Mnl 1 and Hind III enzymes. The phenotypes of the donors were confirmed by activated protein C resistance test (Coatest, IL SpA, Milano, Italy). EBV was added to the blood samples to produce immortalised lymphoblastoid cell lines. Master cell

stocks were accumulated and stored to ensure continual future supplies of the same cells. gDNA samples were extracted from cell pellets, using a Puregene DNA purification kit (Gentra Systems, Minneapolis, USA). gDNA samples extracted using the same purification procedure from EBV transformed cell lines have been shown to be non-infectious in an EBV infectivity assay. However, these candidate materials should still be handled with care and according to laboratory safety precautions for biological materials. The purity of the extracted DNA was confirmed by optical density and agarose gels. The samples were then freeze-dried in sealed glass ampoules[19]. The concentration of gDNA in each ampoule was estimated by OD to be approximately 100µg/mL. The pH of the reconstituted candidate materials was measured and all 3 were found to be within 7.6 to 7.9. The genotypes of the freeze-dried samples were further analysed and confirmed by RFLP and allelic discrimination (Taqman technology). Table 1 shows the product summary for the three materials in the panel.

Table 1 Product summaries for the proposed 1st International Genetic reference panel for Factor V Leiden, Human gDNA: 03/254, 03/260, 03/248

NIBSC Code	**03/254 FV Wild Type**	**03/260 FVL Homozygote**	**03/248 FVL Heterozygote**
Presentation	Sealed Glass din ampoules		
No of ampoules available	3500	3500	3500
Excipient	Trehalose 5mg/ml in Tris/EDTA buffer		
Coefficient of variation of the fill (%)	0.11	0.08	0.61
Residual moisture after lyophilisation and secondary desiccation (%)	1.83	0.77	1.56
Mean dry weight (g)	0.0028	0.0028	0.0030
Mean oxygen content (%)	0.855	1.381	1.305

PARTICIPANTS

Forty-six clinical laboratories agreed to participate in the study and results were returned by 41 laboratories from 16 different countries. Each laboratory has been assigned a code number which does not reflect the order of listing in Appendix I which shows the list of invited participants.

STUDY DESIGN

Eighteen coded samples of the panel of three gDNA materials were sent to each laboratory. Each laboratory was asked to perform their routine genotyping test for factor V Leiden and that the coded samples should be genotyped in groups of 6 on 3 separate days as indicated on the results sheet, using different reagents or different operators if possible. If the participating laboratory routinely used

more than one genotyping method, those methods could be carried out in the same way on the same sets of coded samples.

Overall findings of each sample and raw data eg photographs of gels, amplification plots were to be returned together with full detail of techniques used, any in house or commercial controls and reasons for failure of any of the samples genotyped.

METHODS

The methods used by the participants are listed in Appendix II. Overall, there were 18 different techniques and also 14 different variations of the RFLP employed by the 41 laboratories. As shown in Table 2, the RFLP and melting curves analysis using the Roche Lightcycler were the 2 most frequently used methods amongst the participants of the study. There were 7 different commercial kits employed in the study (Table 3), the remainder used in-house methods.

Table 2 Techniques used by the participants

Techniques	No of Labs
14 different variants of RFLP	21
Melting Curves, using Lightcycler	8
Melting Curves, using Rotogene	1
Allelic discrimination, using ABI Taqman	4
SSCP	2
PCR/SSOP reverse hybridisation	1
Multiplex ARMS PCR	1
ASAPCR; multiplex	1
PCR heteroduplex	2
Mutagenically separated PCR detected on (Genescan ABI 310)	1
Mutagenically separated PCR detected by gel	1
Nanogene array	1
Invader assay	1
Fluorescence based multiplex	1
Pyrosequencing	1
Transgenomic DHPLC	1
DNA strip Thrombotype (Hain Lifesciences)	1
Pronto Thromborisk	1
Allele specific multiplex PCR (Technoclone venous thrombosis genotyping kit)	1

Table 3 Commercial Kits

Commercial Kit	No of labs
Roche FV Leiden kit	8
Helena Biosciences FV mutation detection kit	1
Nanogene array	1
Invader Assay	1
Hain Lifesciences DNA STRIP Thrombotype	1
Pronto Thromborisk	1
Technoclone venous thrombosis genotyping kit	1

A list of references related to the RFLP methods used by the participants is provided in Appendix III. Of the 41 participants, 28 provided information on the primers and probes that they have used and these are listed in Appendix IV. The primers and probes were aligned with the FV sequence contained in the NCBI database (clone HS86F14). The factor V Leiden mutation is at position 62,936 and the region covered by the 26 laboratories ranged from position 55409 to 63227. Appendix V shows the relative positions of the primers and probes.

RESULTS

Testing carried out by participants

Laboratories were asked to carry out the testing on 3 separate days and 2 labs commented that they were unable to do this. Fourteen labs were able to use more than one batch of reagents and the testing was carried out by more than one operator in 12 labs.

Expected correct results

The 18 test samples comprised just 3 preparations and the following results were expected (Table 4):

Table 4 Expected Results

	Factor V Leiden		
	absent	**heterozygous**	**Homozygous**
Sample 1	✓		
Sample 2		✓	
Sample 3			✓
Sample 4			✓
Sample 5		✓	
Sample 6	✓		
Sample 7		✓	
Sample 8	✓		
Sample 9			✓
Sample 10		✓	
Sample 11			✓
Sample 12		✓	
Sample 13		✓	
Sample 14	✓		
Sample 15		✓	
Sample 16			✓
Sample 17			✓
Sample 18	✓		

Results returned by the participants

- 35 labs returned results identical to those above using one or more techniques: 4 labs (lab no. 2, 36, 38, 42) used two different techniques for all samples and one lab (lab 26) used 3 techniques for all samples, all with identical results. Lab 28 used 2 techniques; RFLP with Hind III gave results identical to those above but when using RFLP with MnI 1 it was not possible to determine a result with one sample (see table 6 below). Lab 39 used one technique for samples 1-6 (RFLP) and

another technique for samples 7-18 (ASAPCR) and all results were correct. Lab 1 used one technique for all samples (Roche Lightcycler) and two other techniques (heteroduplex and RFLP) for samples 7-12 only, all with correct results.

- 2 of the 35 labs returning correct results commented that some samples gave weaker than expected results as follows:

Table 5 Laboratories with correct but weaker than expected results

Lab No.	Technique	Weak results
23	Mutagenic separated PCR	Samples 1-6
34	RFLP	Samples 5, 10, 13 & 15

- 5 labs were unable to obtain results for some or all of the samples, as shown below; however, all the results they were able to determine were correct.

Table 6 Laboratories unable to obtain results with some samples

Lab No.	Technique	Samples not determined	Laboratory comment
28	RFLP	13	Insufficient PCR product
29	RFLP	1-18	? samples too concentrated
31	RFLP	13-18	No PCR product
44	Hain Thrombotype	7-12	Dilution error
45	Pronto Thromborisk	7-18	Insufficient test kit reagents

- 2 labs had 3 errors each, as follows:

Table 7 Laboratories with errors in results

Lab No.	Technique	Sample	Laboratory result	Expected result
20	RFLP	1	homozygous	absent
		17	absent	homozygous
		18	homozygous	absent
21	RFLP	3	heterozygous	homozygous
		4	heterozygous	homozygous
		16	heterozygous	homozygous

DISCUSSION

Forty-one laboratories took part in this international collaborative study to evaluate the suitability of the proposed panel of gDNA samples as the 1st International Genetic Reference Panel for Factor V Leiden. The study was designed to determine how well the panel would perform in a large number of laboratories, using a wide variety of methods. The participants used 18 techniques with different underlying principles. Within these 18 techniques there were a total of 32 different individual protocols. In order to assess the consistency of the panel's performance, the participants

were also requested to carry out the study over a period of three days and using different operators when possible.

With the exception of 2 laboratories that returned erroneous results, one laboratory that was unable to determine the genotypes of any of the samples and 4 participants that were only able to genotype some of the samples, all other participants were able to genotype the coded samples correctly. There was no clear correlation between the wrong results and any particular samples. In total, 859 genotyping tests were carried out in this study. With 6 incorrect results, the error rate was calculated to be 0.7%. All errors were made in two laboratories using RFLP, however, RFLP was used by more than half of the participants and it is not possible conclude that the errors are related to the technique employed. In addition, only 2.9% of the genotyping tests gave incomplete or inconclusive results. These data indicate that the panel was suitable for use as positive references for wild type, heterozygote and homozygote factor V Leiden.

COMMENTS FROM THE PARTICIPANTS AND THE MEMBERS OF THE SCIENTIFIC AND STANDARDIZATION COMMITTEE (SSC) OF THE INTERNATIONAL SOCIETY ON THROMBOSIS AND HAEMOSTAIS (ISTH)

Three of the 8 laboratories using the "Lightcycler" found that the melting temperatures for the panel of samples were 2°C lower than their in-house and the manufacturer's controls, while one laboratory found the melting temperature to be less than 0.5 °C lower only for some of the samples. Nonetheless, all laboratories using the Roche Lightcycler kit were able to identify the genotypes of the samples correctly. In-house investigations at NIBSC have indicated that this discrepancy may be due to the amount of DNA added to the system and that the excipient has no effect on the "Lightcycler" method.

Two laboratories found "weak bands" for some of the samples, however, this was not related to any one specific sample. One laboratory using the Allele discrimination by Taqman technology mentioned that the samples were giving "more higher and lower outlying signals" than own in-house controls. Other laboratories using the same techniques have not reported similar findings.

No other specific comments were made by the participants in relation to the performance of the panel using their methods of detection.

Twenty-nine out of the 41 participants agreed with the proposal that this panel of 3 gDNA should be recommended as the 1st International Genetic Reference Panel for Factor V Leiden. The other 12 participants have not sent any comments or responses. The ISTH/SSC Subcommittee on Plasma coagulation Inhibitors have also approved the recommendation and this was subsequently endorsed at the business meeting of the SSC on the 19 June 2004.

DEGRADATION STUDY

Preliminary accelerated degradation study on samples stored at +56°C and +45°C were carried out. When compared with the -150°C samples after 4 months storage for 03/248, 3 months storage for 03/260 and 2 months storage for 03/254, there was no detectable degradation in any of the samples by quantitative PCR. This was confirmed by agarose gel electrophoresis, where there was no difference in the electrophoretic profiles of the -150°C, +56°C and the +45° samples. No smearing was seen with any of the samples. The integrity of the high molecular weight band shown by the -150°C samples also indicates that there is hardly any degraded gDNA in all 3 candidate materials. Continual real time degradation study of the -20°C samples against the -150°C and further accelerated degradation study at elevated temperature will be carried out to monitor the stability of the panel.

CONCLUSIONS AND RECOMMENDATION

The results have shown that the panel of three gDNA samples evaluated in this international multi-centre study is suitable as reference materials for laboratories carrying out genotyping for factor V Leiden. It is therefore recommended that the 3 human gDNA preparations:

03/254 (FV wild type)
03/260 (FVL homozygote)
03/248 (FVL heterozygote)

be put forward to the Expert Committee for Biological Standardisation (ECBS) of the World Health Organisation (WHO) to be established as the 1st International Genetic Reference Panel for Factor V Leiden, Human gDNA.

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