



ORIGINAL ARTICLE

Genetics of haemostasis

A. C. GOODEVE,* D. J. PERRY,†‡ T. CUMMING,† M. HILL,† I. JENNINGS,† S. KITCHEN,† I. WALKER,† E. GRAY,§ G. R. JAYANDHARAN¶ and E. TUDDENHAM**

*Sheffield Diagnostic Genetics Service, Sheffield Children's NHS Foundation Trust and Haemostasis Research Group, Department of Cardiovascular Science, Sheffield University Faculty of Medicine, Dentistry and Health Sheffield, Sheffield, UK;

†on behalf of UK NEQAS for Blood Coagulation - Haemophilia Genetic Analysis Specialist Advisory Group, Sheffield, UK;

‡Department of Haematology, Addenbrooke's Hospital, Cambridge, UK; §National Institute for Biological Standards and

Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire, UK; ¶Department of Haematology and Centre for Stem Cell Research, Christian Medical College, Vellore, India; and **The Katharine Dormandy Haemophilia Centre and Thrombosis Unit, Royal Free National Health Service Trust, London, UK

Summary. Congenital defects of platelets or plasma proteins involved in blood coagulation generally lead to bleeding disorders. In some of these disorders, patients with a severe phenotype are prone to spontaneous bleeds with critical consequences. This situation occurs more commonly in haemophilia A and haemophilia B and to a certain extent in severe forms (type 3) of von Willebrand disease. Defects in other plasma coagulation proteins and platelet factors are relatively rare, with an incidence of ≤ 1 : 1–2 million. Molecular genetic studies of the human coagulation factors, especially factors VIII and IX, have contributed to a better understanding of the biology of these genetic disorders, the accurate

detection of carriers and genetic counselling, and have also fostered new therapeutic strategies. This article reviews the evolution of genetics over the last five decades as a tool for bleeding disorder investigations, the recent advances in molecular techniques that have contributed to improved genetic diagnosis of this condition, and the development and utility of proficiency testing programmes and reference materials for genetic diagnosis of bleeding disorders.

Keywords: bleeding disorders, genetic diagnosis, haemophilia, mutation, mutational analysis, quality assurance

Blood clotting is a host defence mechanism that, in parallel with the inflammatory responses, not only helps to protect the integrity of the vascular system but also promotes repair after tissue injury. This process involves a series of orderly steps including components of the vasculature, platelets (primary haemostasis) and coagulation proteins (secondary haemostasis), leading to the formation of a platelet plug and culminating in the formation of a stable fibrin clot. Congenital defects of platelets or plasma proteins involved in this process generally lead to lifelong bleeding disorders [1,2].

Haemophilia A and haemophilia B, both of which are X-chromosome linked and caused by a defect of coagulation factor (F) VIII or FIX, are more common [3–5]. Other bleeding disorders, with the exception of von Willebrand disease, are relatively rare (Table 1).

Molecular genetic diagnosis of bleeding disorders remains an important and integral part of the evaluation of this condition. There are two different approaches to the genetic evaluation of bleeding disorders: analysis of single nucleotide polymorphism (SNP) or microsatellite short tandem repeat (STR) markers in the gene of interest to track the defective chromosome in the family (linkage analysis), or identification of the disease-causing mutation in the patient's coagulation factor gene (direct mutation detection) [6,7]. Before embarking on genetic testing, it is imperative that detailed clinical evaluation and conclusive phenotypic diagnosis be available.

In this review, the authors trace the evolution and the applications of molecular genetics in bleeding disorders. The current protocols available for genetic testing is a convergence of intense research and development of genetic tools over the last 50 years (*Prof. Tuddenham*) and which has benefited immensely from the availability

Correspondence: Prof Edward Tuddenham, The Katharine Dormandy Haemophilia Centre and Thrombosis Unit, Royal Free National Health Service Trust, University College, London, UK. Tel.: +207 794 0500 (ext. 34142); e-mail: e.tuddenham@ucl.ac.uk

(Or)

GR Jayandharan, Department of Haematology/Centre for Stem Cell Research, Christian Medical College, Vellore-632004 Tamil Nadu, India.

Tel.: +416 2283562; Fax: +416 2226449; e-mail: jay@cmcvellore.ac.in

Accepted after revision 15 February 2012

Table 1. General characteristics of bleeding disorders.

Disease/deficiency	Prevalence	Gene symbol	Gene location on chromosome	No. of reported mutations*
Bernard Soulier syndrome	1:1 million	<i>GPIBA</i>	17p12	36
		<i>GPIIB</i>	22q11.2	24
		<i>GP9</i>	3q21	21
Glanzmann thrombasthenia	1:1 million	<i>ITGA2B</i>	17q21.32	147
		<i>ITGB3</i>	17q21.32	110
Wiskott-Aldrich syndrome	1:1 million	<i>WAS</i>	Xp11.4-p11.21	369
Von Willebrand disease		<i>VWF</i>	12p13.3	522
Fibrinogen	1:1 million	<i>FGA</i>	4q28	100
		<i>FGB</i>	4q28	59
		<i>FGG</i>	4q28	85
Prothrombin	1:2 million	<i>F2</i>	11p11	57
Factor V	1:1 million	<i>F5</i>	1q23	142
Factor V + VIII	1:1 million	<i>LMAN1</i>	18q21.3-q22	34
		<i>MCFD2</i>	2p21	19
Factor VII	1:500 000	<i>F7</i>	13q34	263
Factor VIII	1:5000	<i>F8</i>	Xq28	2307
Factor IX	1:25 000	<i>F9</i>	Xq27.1-q27.2	1104
Factor X	1:1 million	<i>F10</i>	13q34	112
Factor XI	1:1 million†	<i>F11</i>	4	202
Factor XIII A	1:1 million	<i>F13A1</i>	6p25.3-p24.3	114
		<i>F13B</i>	1q31-q32.1	19

*Data from Human Gene Mutation Database, <http://www.hgmd.org/>. Accessed February 4, 2012.

†Homozygous factor XI deficiency.

of a vast repertoire of bio-informatics and molecular biology tools over the last decade or so (*Dr. Anne Goodeve*). With a steady growth in the number of laboratories that offer genetic testing for disorders of haemostasis worldwide, the availability of rigorous external quality assessment programmes (*Dr. David Perry*) and reference materials to run such programmes (*Dr. Elaine Gray*) have helped to maintain the quality and integrity of reporting data during the genetic testing of various bleeding disorders.

Development of genetics as a tool in bleeding disorder investigations during the 50-year development of the WFH

Edward Tuddenham

Since 1962 is the starting point of this short history, one asks oneself, 'What was it like back then?' Personally I had been accepted into Westminster Medical School and was studying mathematics during what is now called 'the gap year'. Although I was in London, the famous 60s passed me by almost completely. Genetics as a science was still in its formal era as defined by Haldane in the Croonian lecture of 1948. He predicted with astonishing prescience that DNA and biochemical studies would take over; yet despite the publication of the structure of DNA by Watson and Crick in 1952 and subsequent progress on the genetic code, what came to be called molecular genetics had

not by 1962 penetrated beyond the specialized laboratories working on nucleic acids, much less to the clinical genetics departments. Linkage of phenotypes was still the only way to track a gene in kindred segregating a genetic disorder such as haemophilia. This had already been achieved in 1937 by Haldane and Bell, who linked haemophilia to colour blindness, the first definite linkage of any two traits in man. Of course this was not of much practical use, but by 1962, no progress had been made in defining haemophilia beyond separating haemophilia A from haemophilia B by specific coagulation factor assays. Very slowly, molecular genetics began to penetrate clinical genetics. But the first major advance in haemophilia genetics after 1937 was the demonstration by Zimmerman and Ratnoff in 1970 that the ratio of FVIII activity to FVIII-related antigen was predictive of carrier status for haemophilia A. I became interested in haemophilia in 1969, and in 1976, I set out to purify factor VIII. What follows is my journey into the genetics of haemophilia A, during which I and my colleagues made clinically relevant advances based on the molecular genetics of the *F8* gene. A parallel journey was undertaken by Brownlee, Gianelli and others studying haemophilia B and the *F9* gene. The story of von Willebrand disease genetics is highly complicated and can only be done justice in a separate essay.

My first foray into linkage, published in 1984, was to show that a polymorphic DNA probe DX13 was linked to haemophilia A and could be used for carrier determination, albeit with the caveat that meiotic crossover could vitiate the linkage and therefore accuracy of the prediction [8]. The same year with Genentech, we had cloned the *F8* gene and established the complete sequence at both protein and cDNA levels [9,10]. The following year, Jane Gitschier, who had mapped the *F8* locus [3], found a polymorphism in the region of exon 18, which we quickly showed could be used for allele tracking in potential carrier females of haemophilia A [11]. This polymorphism was immediately put to work in the antenatal diagnosis of haemophilia A by chorionic biopsy analysis [12]. The *F8* locus proved to have very few polymorphisms susceptible to analysis by restriction fragment length polymorphism analysis (RFLP), the only practical tool we had to detect them at that time. One further polymorphism was found with the help of the Genentech team, the so-called *XbaI* RFLP, which is located in intron 22 of *F8* [13]. All these RFLPs were laboriously analysed by means of Southern blotting with labelled probes from the *F8* gene. Even so, with just three RFLPs, many females were uninformative. So, with John McVey and my new research group at Northwick Park, we set out to find a different type of polymorphism created by short tandem repeats whose number varied (STR). In 1991, we found a highly informative STR in intron 13 [14], which together with a second STR in intron 22

discovered in 1994, gave an informative result for over 90% of potential carriers [15].

The next advance was in methods for sequencing of the coding region of *F8* based on PCR, to find the underlying mutations [16]. This opened up the possibility of having a unique marker for the defective allele in every family segregating haemophilia A. At first, with the expensive and laborious sequencing methods available, it was necessary to screen using techniques that could show altered behaviour in a small fragment, which was then sequenced [17]. The advent of first generation sequencing machines made it feasible to sequence an entire coding region without a screening step. It meant that we could find a mutation in one-to-two weeks. As a result of this sequencing, it soon became evident that there was no plausible disease-causing mutation in about half the severely affected cases. Jane Gitschier returned to the *F8* gene to show that, in such cases, there was an inversion involving either of two copies of an intronic gene *F8A* located within intron 22 and an extragenic copy of *F8A* located 400 kb upstream [18].

The international haemophilia A database, which I started in 1991 [19] to improve understanding of the correlation between mutation and phenotype, went online in 1996 [20]. From a critical analysis of the mutations published up to that date, we discovered that some mutations had a highly variable phenotype and that there was a stronger risk of inhibitor development for some types of mutation than others [21]. The database has grown steadily and as of 2004 listed over 1000 unique mutations. This is set to increase massively with the next update and overhaul of the site in 2012.

The most recent technical advance in detection of foetal DNA in maternal blood now allows the status of a foetus to be determined as early as week 11 of gestation [22]. In the next 50 years, genetic tools will come to dominate not only diagnosis but treatment of the haemophilias, which after all are the classic example of genetic disorder in man.

Genetic analysis in bleeding disorders

Anne C. Goodeve

Many recent enhancements to processes used in genetic analysis of inherited bleeding disorders are available. They are reviewed here following the pathway from patient referral to reporting of results.

Sample analysis

Computer-based laboratory information management systems (LIMS) can provide a complete system of 'paperless' sample management. All patient referral documentation can be scanned and stored electronically,

work lists can be generated for testing to be undertaken, and results can subsequently be recorded within the LIMS. Genomic DNA can be prepared from blood and other tissues using a variety of automated extraction procedures. Bar-coding of individual samples and of the plates in which they are analysed facilitates recording storage location and ensures that the correct samples are transferred between containers during analysis.

Several genetic analysis techniques are available, generally utilizing genomic DNA as template. PCR amplification of promoter, exons plus flanking intron and 3' untranslated regions can be streamlined through use of single amplification conditions, with some DNA polymerases facilitating amplification of a range of template sizes and GC contents under single amplification conditions. PCR primers tailed with common sequences (e.g. m13) facilitate DNA sequence reaction set up, enabling any amplicon from any gene to be sequenced using single forward and reverse primers. Mutation scanning techniques such as high resolution melt curve analysis (HRM) [23] are also amenable to high throughput analysis. Robotic sample processing eliminates inter-user variation in pipetting, resulting in more consistent DNA sequence whilst enhancing sample throughput. Following DNA sequencing, application of sequence analysis software enables rapid identification of variants that differ from the reference sequence (RefSeq). Large deletions and duplications can be identified using multiplex ligation-dependent probe amplification (MLPA) [24,25], micro-array or mutation-specific gap-PCR [26]. *F8* intra-chromosomal inversions amplified through long or inverse PCR can be analysed using gel electrophoresis or, for smaller amplicons, by fragment sizing using a DNA sequencer. Previously 'missing' deep intronic mutations that affect splicing can be detected by reverse transcription (RT)-PCR from mRNA, gel electrophoresis and DNA sequencing [27,28]. Sequence-variant interpretation software integrates pathogenicity prediction tools for amino acid substitutions and for splice variants along with literature searching for previously reported variants. For amino acid substitutions, these are based on different algorithms for their potential impact on protein structure/function using assessment of physical properties, along with their evolutionary conservation. Mutations potentially affecting splicing are analysed by panels of algorithms analysing presence and relative strength of splice motifs. Locus-specific mutation databases, the Human Gene Mutation Database (HGMD) [29], and the single nucleotide polymorphism database (dbSNP) [30] catalogue and help facilitate pathogenicity interpretation of previously reported variants. Newly recognized sequence variants underneath PCR amplification primers can be sought with each new release of dbSNP using tools including SNPcheck [31]. Primers can then be redesigned where necessary using online software to prevent mono-allelic amplification resulting in allele dropout and mutations potentially being missed. [32] Reference genetic materials

are available for common mutations through the UK National Institute for Biological Standards and Control (NIBSC) [33] and can be used to validate assay performance. LIMS use can facilitate reporting of the results of genetic testing to the referring clinician through ready availability of all patient documentation on the computer screen and through integration of standard report templates with specific details of testing undertaken and its results. Reports generated can be passed between scientists electronically for content verification, and where secure electronic document transfer is possible, for electronic reporting back to the referring clinician without the requirement to print and post the report.

Standardization

Guidelines from bodies including the UK Clinical Molecular Genetics Society, the European Molecular Genetics Quality Network (EMQN), and the Swiss Society of Medical Genetics recommend standard practice in several areas including validation and verification of molecular genetic tests, DNA sequencing, quality control and pathogenicity prediction of sequence variants as well as for disease-specific issues. EuroGenTest maintains a guideline listing [34]. Laboratory accreditation to national or international standards ensures that common standards of practice are maintained, while quality management software facilitates organization and regular review of laboratory management and standard operating procedure documents. Use of standard Human Genome Organisation Gene Nomenclature Committee (HGNC) gene names [35], along with Human Genome Variation Society (HGVS) sequence nomenclature [36] and reference to a specified RefSeq, reduces errors in documenting variants identified by different laboratories. External quality assessment (EQA) for genetic analysis is available for a limited number of bleeding disorders (currently haemophilia A, haemophilia B and von Willebrand disease) through bodies including the UK National External Quality Assessment Survey (NEQAS) for Blood Coagulation. Participation in regular surveys leads to improvement in clerical and genotyping accuracy and in the completeness of sequence variant interpretation in genetic analysis reports [37]. Generic EQA for DNA sequence analysis and interpretation is also available through bodies including EMQN. Sharing best laboratory practice and provision of backup laboratory analysis when problems arise is made possible by participation in laboratory networks e.g. the UK Haemophilia Centre Doctors' Organisation (UKHCDO) Genetic Testing Network [38].

The future

Next generation DNA sequencing will shortly start to contribute to identification of exonic and currently

'missing' intronic and transcriptional sequence variants, enhancing the range of bleeding disorders that can readily be analysed, while helping to reduce analysis costs.

The NEQAS Haemophilia Molecular Genetics Quality Assurance Scheme

David J Perry, Tony Cumming, Anne Goodeve, Marian Hill, Ian Jennings, Steven Kitchen and Isobel Walker

Molecular genetic analyses in families with haemophilia and other inherited bleeding disorders is a common laboratory investigation. The results of genotypes are unequivocal with no borderline values, but a failure to correctly identify a mutation or to misinterpret its significance can have major implications for an individual, his/her family and offspring. In contrast to phenotypic testing in which strict quality control is adhered to, in the field of haemophilia, molecular genetic testing, many/most laboratories do not appear to participate in any external quality assurance (EQA) schemes. However, experience from other genetic EQA schemes has highlighted that errors, both in the correct identification of mutations and in their subsequent interpretation, may occur [39–42].

The UK NEQAS Scheme for molecular genetic testing in haemophilia

In 1998, the UK National External Quality Assessment Scheme for Blood Coagulation (UK NEQAS BC) established a pilot quality assurance scheme for molecular genetic testing in haemophilia. The scheme was designed to assess genotyping, clerical accuracy and the interpretation of genetic analyses in individuals with inherited bleeding disorders. In the original pilot schemes, the presence or absence of the *F8* intron 22 inversion was examined. Results from three initial surveys highlighted problems with the quality of samples when used to screen for the intron 22 inversion and in part reflected difficulties with these analyses by Southern blot analysis or by long-range PCR at this time. The scheme was re-launched in 2003 and subsequent exercises have included *F8* linkage studies as well as mutations within the *F8*, *F9* and *VWF* genes [37].

The Scheme

The scheme is open to laboratories from all countries and currently 22 laboratories, primarily but not exclusively from Europe, participate. Participants in the scheme are provided with a clinical scenario (Fig. 1) and either whole blood or DNA isolated from previously established cell lines and asked to seek a familial



UK NEQAS FOR BLOOD COAGULATION
UK NATIONAL EXTERNAL QUALITY ASSESSMENT SCHEMES

Floor 3, Pegasus House
463a Glossop Road
Sheffield S10 2QD

E-mail: neqas@coageqa.org.uk

Professor ID Walker (Director)
Mr TAL Woods (Manager)
Tel: +44 (0)114 267 3300
Fax: +44 (0)114 267 3309

20 October 2011

Participant No. XXX

**Haemophilia Genetic Analysis External Quality Assessment
Exercise 19**

Dear Colleague

Please find enclosed two vials containing material for *F8* gene analysis, with details as described below. If you have any queries regarding this distribution, please contact the UK NEQAS for Blood Coagulation office (0114 267 3300), or email Ian Jennings (i.jennings@coageqa.org.uk).

The vials contain liquid gDNA for Haemophilia genetic analysis. Each vial contains 25ul of DNA; The concentration of DNA in sample **HG11:02** is 580ng/ul, and in sample **HG11:03** is 700ng/ul.

Exercise 19

Referring Hospital: Fairview Hospital, Barrow in Furness, Cumbria, LA14 2PW
Referring Consultant: Dr D.I. Apson, Consultant Haematologist

Isaac Glenfarclas has recently been diagnosed with severe haemophilia A [FVIII:C <1 IU/dL.]

DNA samples are available on Isaac Glenfarclas and his sister Fenella Glenfarclas. Fenella has a FVIII:C of 129 IU/dL and wishes to know if she is a carrier of haemophilia A or not.

Name	DOB	Hospital Number	Sample Number
Isaac Glenfarclas	11/6/2007	A893461	HG11:02
Fenella Glenfarclas	21/8/1991	A547689	HG11:03

1. Please undertake *F8* intron 22 inversion analysis to establish whether this is the cause of Isaac Glenfarclas' low FVIII:C levels, and to determine his sister Fenella's carrier status.

2. Prepare a report using your standard format, **removing any details that identify yourself or your laboratory**. Please include your NEQAS participant number (shown at the top of this page) at the top of your report.

3. Please return your results to Ian Jennings, by post to: *Floor 3, Pegasus House, 463a Glossop Road, Sheffield S102QD, UK* or preferably as email attachments (i.jennings@coageqa.org.uk) by **2nd December 2011** at the latest; analysis of the results from this survey will be reported to participants as soon as possible after this date.

Specialist Advisory Group

Dr A Cumming, Dr A Goodeve, Dr I Jennings, Dr S Kitchen, Dr M Hill, Dr D Perry (Chairman)

Fig. 1. Clinical data provided to the laboratories participating in hemophilia genetic analysis EQA module.

mutation in a limited part of the relevant gene, to report on the mutation in the patient, its presence/absence in relative(s) and the implications for each individual analysed. Reports are assessed in three areas: clerical accuracy, genotyping and interpretation (Fig. 2). Two exercises per annum are circulated with a 6-week turnaround time. Reports are assessed by a panel of scientists/clinicians with expertise in this area. To date, 19 exercises have been circulated covering a wide

variety of mutations [including inversions events, missense and nonsense mutations, and small deletions] within the *F8*, *F9* and *VWF* genes.

Results to date

A laboratory report is a summary of any investigations that have been performed and the subsequent interpretation of these results in the light of any phenotypic and

Haemophilia Molecular Genetics — UK NEQAS (Blood Coagulation)						
Laboratory ID Number: HG11:02		Exercise: 19		Circulated: 20/10/2011		
LAB ID NUMBER	CLERICAL		GENOTYPING		INTERPRETATION	
1	Full Name DOB or Hospital Number	1.5	Technique/Extent of analysis	0.25	Consistent with the observed phenotype [i.e. severe haemophilia A]	1.5
EXERCISE NUMBER 19	Clinical Question Disease What is the clinical question & the reason for the investigation?	0.25	Clear and unambiguous identification of mutation	1.5		
	Referring Hospital & consultant	0.25	Homozygous, Heterozygous or Hemizygous	0.25	Answers the clinical questions Can be used in family studies	0.5
Comments						
Score ^{1,2}	(Max = 2)		(Max = 2)		(Max = 2)	

Scoring based upon CMGS guidelines ¹Maximum overall score = 6 ²A score of <1 in any column equates to a fail.

Fig. 2. Reporting format distributed to participants of the haemophilia EQA program.

family data that were provided. It is important to remember that in many cases, laboratory reports will be read and the contents acted upon by individuals who may not be experts in the field of molecular haemostasis and therefore the reports should be easily interpretable and 'stand alone.' Novel mutations should, for example, include an indication as to why they are considered pathogenic, and how the possibility that they could represent polymorphic variants was excluded. In addition, mutations should adhere to conventional methods both for nomenclature and for numbering [36].

In the NEQAS scheme, scoring for each report (Fig. 2) is based on:

1. Clerical accuracy: was the patient correctly identified, was the clinical question, the disorder and its severity clearly stated?
2. Genotyping: what methods were employed for the analyses and what was the extent of the analysis; was the presence (or absence) of a mutation correctly identified and the predicted consequences of this mutation on protein structure (briefly) explored; was the mutation present in a homozygous, heterozygous or hemizygous form?
3. Interpretation: Was the interpretation based on all data provided and derived from the results of mutational analysis; was this interpretation accurate and did it answer the clinical question?

The assessment template is similar but slightly different for each exercise and the key points for each exercise are decided in advance of the assessment. All reports are scored anonymously, but a unique laboratory number is assigned to each laboratory so that rapid identification

of consistently poorly performing laboratories can be identified and highlighted to the NEQAS BC Steering Committee.

Of the 19 exercises that have been circulated, common reasons for failure or for losing points in the assessment include:

1. A failure to include sufficient unique identifiable patient data.
2. A failure to correctly identify the mutation.
3. Incorrect numbering of the mutation.
4. Incorrect interpretation of the significance of the mutation for the patient and their family members.

Summary

The aim of any EQA scheme is to highlight problems and deficiencies in laboratory procedures. In the UK, laboratories undertaking genetic studies in patients with inherited bleeding disorders are required to participate in an EQA scheme and it is a requirement for membership of the UKHCDO Haemophilia Laboratory Network and for accreditation through CPA (Clinical Pathology Accreditation (UK) Ltd.).

Since its inception, the NEQAS QA Scheme for Haemophilia Genetics has seen a significant improvement in the quality of laboratory reports. Reports are confined to a single page; participants now regularly include essential information, adhere to international recommendations on gene and mutation nomenclature and include relevant reference sequences and literature references. Reports are more 'stand alone' so that genetic information and its interpretation may follow

the patient more readily. The scheme has therefore improved consistency of reporting standards across participating laboratories.

The value of this scheme is highlighted by the last exercise (Exercise 19) in which four laboratories, several of which were new participants to the scheme, failed to correctly identify the presence of a *F8* intron 22 mutation in a heterozygous female. Their participation in the EQA scheme and the subsequent feedback will help incorporate corrective measures in their genetic testing protocols.

Reference materials for genetic tests in haemophilia and allied bleeding disorders

Elaine Gray

The frequency of genetic testing is increasing and the accuracy of these tests is of paramount importance to the diagnosis and treatment of patients and the counselling of affected families. Haemophilia A is a hereditary genetic bleeding disorder occurring in about 1 in 5000 male births, with intron 22 inversion mutation of the *F8* gene accounting for 50% of cases of severe haemophilia A. Genetic analysis of the intron 22 inversion is challenging, involving technically demanding methods such as Southern blotting and long-range PCR [43,44].

External quality assurance schemes have shown that errors in genotyping for this mutation do occur [37]. Most laboratories use as their in-assay control DNA samples extracted from patients known to carry the intron 22 inversion mutation. However, these are not well characterized and are usually only available in limited amounts. Few certified and commercial genetic reference materials for haemophilia and other bleeding disorders are available.

The National Institute for Biological Standards and Control (UK), under the auspices of the World Health Organisation (WHO), therefore investigated the possibility of providing a panel of robust and stable genetic reference materials to improve the accuracy of genotyping for the intron 22 mutation. This panel of four preparations was produced from genomic DNA (gDNA) extracted from immortalized cell lines produced by Epstein-Barr virus transformation of lymphocytes from blood samples of consented donors. The samples were obtained from two normal individuals (male and female), an intron 22 inversion-positive female carrier and an intron 22 inversion-positive male. An international collaborative study involving fourteen laboratories, employing a total of six

different methods with three different underlying principles, evaluated the suitability of the proposed panel of gDNA samples as the 1st International Genetic Reference Panel for Hemophilia A Intron 22 Inversion, Human gDNA. With the exception of one laboratory that returned erroneous results, all other participants were able to genotype every coded sample correctly. All errors concerned the intron 22 inversion-positive female carrier DNA, which was genotyped as a normal individual. With three incorrect results in 166 tests, the overall error rate for this study was 1.8%. These data indicate that errors in genotyping can occur and the vast majority of laboratories can use these materials to obtain the correct result. They also show that the panel is suitable for use as reference materials for normal females, normal males, intron 22 inversion-positive female carriers, and intron 22 inversion-positive affected males. With the exception of one laboratory that did not run known patient samples as in-assay control, all other labs employed appropriate assay control, thereby confirming the commutability of the panel.

In 2008, the World Health Organisation (WHO) established this stable reference panel of genomic DNA (gDNA) (NIBSC Code, 08/160) to support the genetic testing of intron 22 mutation [45]. This panel has been distributed worldwide and has proven to be useful in aiding laboratories to set up and validate their methods. The success of this panel establishes the basis for the future production of genetic reference materials for bleeding disorders.

Conclusions

With the advent of molecular genetic techniques, it is obvious that several aspects of care for patients with bleeding disorders have improved substantially over the last five decades. Along with accurate genetic diagnosis of haemophilia and other bleeding disorders, molecular genetics has further enhanced our understanding of the functional biology of proteins involved in blood coagulation, elucidated the basis of inhibitor development and informed new therapeutic approaches such as development of newer clotting factor concentrates and gene therapy.

Disclosure

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

References

- 1 Peyvandi F, Jayandharan G, Chandy M *et al*. Genetic diagnosis of haemophilia and other inherited bleeding disorders. *Haemophilia* 2006; 12(Suppl. 3): 82–9.
- 2 Mannucci PM, Duga S, Peyvandi F. Recessively inherited coagulation disorders. *Blood* 2004; 104: 1243–52.
- 3 Gitschier J, Wood WI, Goralka TM *et al*. Characterization of the human factor VIII gene. *Nature* 1984; 312: 326–30.
- 4 Yoshitake S, Schach BG, Foster DC, Davie EW, Kurachi K. Nucleotide sequence of the gene for human factor IX (antihemophilic factor B). *Biochemistry* 1985; 24: 3736–50.

- 5 Drayna D, White R. The genetic linkage map of the human X chromosome. *Science* 1985; **230**: 753–8.
- 6 Peake IR, Lillicrap DP, Boulyjenkov V *et al.* Haemophilia: strategies for carrier detection and prenatal diagnosis. *Bull World Health Organ* 1993; **71**: 429–58.
- 7 Peyvandi F. Carrier detection and prenatal diagnosis of hemophilia in developing countries. *Semin J Thromb Haemost* 2005; **31**: 544–54.
- 8 Harper K, Winter RM, Pembrey ME, Hartley D, Davies KE, Tuddenham EGD. A clinically useful DNA probe closely linked to haemophilia A. *Lancet* 1984; **ii**: 6–8.
- 9 Vehar GA, Keyt B, Eaton D *et al.* Structure of human factor VIII. *Nature* 1984; **312**: 337–42.
- 10 Wood WI, Capon DJ, Simonsen CC *et al.* Expression of active human factor VIII from recombinant DNA clones. *Nature* 1984; **312**: 330–7.
- 11 Gitschier J, Drayna D, Tuddenham EGD, White RL, Lawn RM. Genetic mapping and diagnosis of haemophilia A achieved through a Bcl I polymorphism in the factor VIII gene. *Nature* 1985; **314**: 738–40.
- 12 Gitschier J, Lawn RM, Rotblat F, Goldman E, Tuddenham EGD. Antenatal diagnosis and carrier detection of haemophilia A using a factor VIII gene probe. *Lancet* 1985; **i**: 1093.
- 13 Wion K, Tuddenham E, Lawn R. A new polymorphism in the factor VIII gene for prenatal diagnosis of haemophilia A. *Nucl Acids Res* 1986; **14**: 4535.
- 14 Lalloz MRA, McVey JH, Pattinson JK, Tuddenham EGD. Haemophilia A diagnosis by analysis of a hypervariable dinucleotide repeat within the factor VIII gene. *Lancet* 1991; **338**: 207–11.
- 15 Lalloz MRA, Schwaab R, McVey JH, Michaelides K, Tuddenham EGD. Haemophilia A diagnosis by simultaneous analysis of two variable dinucleotide tandem repeats within the factor VIII gene. *Br J Haematol* 1994; **86**: 804–9.
- 16 Antonarakis SE, Kazazian HH, Gitschier J, Hutter P, de Moerloose P, Morris MA. Molecular etiology of factor VIII deficiency in hemophilia A. *Adv Exp Med Biol* 1995; **386**: 19–34.
- 17 Kogan S, Gitschier J. Mutations and a polymorphism in the factor VIII gene discovered by denaturing gradient gel electrophoresis. *Proc Natl Acad Sci USA* 1990; **87**: 2092–6.
- 18 Lakich D, Kazazian HH Jr, Antonarakis SE, Gitschier J. Inversions disrupting the factor VIII gene are a common cause of severe haemophilia A. *Nat Genet* 1993; **5**: 236–41.
- 19 Tuddenham EGD, Cooper DN, Gitschier J *et al.* Haemophilia A: database of nucleotide substitutions, deletions, insertions and rearrangements of the factor VIII gene. *Nucl Acids Res* 1991; **19**: 4821–33.
- 20 Wacey AI, Kembal-Cook G, Kazazian HH *et al.* The haemophilia A mutation search test and resource site, home page of the factor VIII mutation database: HAMSTeRS. *Nucl Acids Res* 1996; **24**: 100–2.
- 21 Schwaab R, Brackmann HH, Seehafer J *et al.* Haemophilia A: mutation type determines risk of inhibitor formation. *J Thromb Haemost* 1995; **74**: 1402–6.
- 22 Tsui NB, Kadir RA, Chan KC *et al.* Noninvasive prenatal diagnosis of hemophilia by microfluidics digital PCR analysis of maternal plasma DNA. *Blood* 2011; **117**: 3684–91.
- 23 Lin SY, Su YN, Hung CC *et al.* Mutation spectrum of 122 hemophilia A families from Taiwanese population by LD-PCR, DHPLC, multiplex PCR and evaluating the clinical application of HRM. *BMC Med Genet* 2008; **9**: 53.
- 24 MRC-Holland. MRC-Holland MLPA homepage. Available at: <http://www.mlpa.com>. Accessed January 28, 2012.
- 25 Rafati M, Ravanbod S, Hoseini A *et al.* Identification of ten large deletions and one duplication in the F8 gene of eleven unrelated Iranian severe haemophilia A families using the multiplex ligation-dependent probe amplification technique. *Haemophilia* 2011; **17**: 705–7.
- 26 Mohl A, Marschalek R, Masszi T *et al.* An Alu-mediated novel large deletion is the most frequent cause of type 3 von Willebrand disease in Hungary. *J Thromb Haemost* 2008; **6**: 1729–35.
- 27 Castoldi E, Duckers C, Radu C *et al.* Homozygous F5 deep-intronic splicing mutation resulting in severe factor V deficiency and undetectable thrombin generation in platelet-rich plasma. *J Thromb Haemost* 2011; **9**: 959–68.
- 28 Castaman G, Giacomelli SH, Mancuso ME *et al.* Deep intronic variations may cause mild hemophilia A. *J Thromb Haemost* 2011; **9**: 1541–8.
- 29 HGMD. The Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff. Available at: <http://www.hgmd.cf.ac.uk/ac/index.php>. Accessed January 28, 2012.
- 30 NCBI. dbSNP. Available at: <http://www.ncbi.nlm.nih.gov/projects/SNP/>. Accessed January 28, 2012.
- 31 NGRL. SNPCheck. Available at: nrgl.manchester.ac.uk/SNPCheckV2/snpcheck.htm. Accessed January 28, 2012.
- 32 Hampshire DJ, Burghel GJ, Goudemand J *et al.* Polymorphic variation within the VWF gene contributes to the failure to detect mutations in patients historically diagnosed with type 1 von Willebrand disease from the MCMDM-1VWD cohort. *Haematologica* 2010; **95**: 2163–5.
- 33 NIBSC. WHO International Genetic Reference Panel for Haemophilia A, Intron-22 inversion. Available at: http://www.nibsc.ac.uk/science/diagnostics/genetic_reference_materials/factor_viii_intron_22_inversion.aspx. Accessed January 28, 2012.
- 34 EuroGenTest. Best Practice Guidelines for Molecular Genetic Testing. Available at: http://www.eurogentest.org/laboratories/info/public/unit1/eqa/molecular_genetics/guidelines.html. Accessed January 28, 2012.
- 35 HGNC. HUGO Gene Nomenclature Committee information homepage. Available at: <http://www.genenames.org/>. Accessed January 28, 2012.
- 36 HGVS. Nomenclature for the description of sequence variations homepage. Available at: <http://www.hgvs.org/mutnomen/>. Accessed January 28, 2012.
- 37 Perry DJ, Goodeve A, Hill M, Jennings I, Kitchen S, Walker I. The UK National External Quality Assessment Scheme (UK NEQAS) for molecular genetic testing in haemophilia. *J Thromb Haemost* 2006; **96**: 597–601.
- 38 UKHCDO. Genetics Working Party. Available at: <http://www.ukhcdo.org/wp/genetics.htm>. Accessed January 28, 2012.
- 39 Dequeker E, Cassiman JJ. Genetic testing and quality control in diagnostic laboratories. *Nat Genet* 2000; **25**: 259–60.
- 40 Tripodi A, Peyvandi F, Chantarangkul V, Menegatti M, Mannucci PM. Relatively poor performance of clinical laboratories for DNA analyses in the detection of two thrombophilic mutations—a cause for concern. *J Thromb Haemost* 2002; **88**: 690–1.
- 41 Mueller CR, Kristofferson U, Stoppa-Lyonnet D. External quality assessment for mutation detection in the BRCA1 and BRCA2 genes: EMQN's experience of 3 years. *Ann Oncol* 2004; **15**(Suppl. 1): 114–7.
- 42 Seneca S, Morris MA, Patton S, Elles R, Sequeiros J. Experience and outcome of 3 years of a European EQA scheme for genetic testing of the spinocerebellar ataxias. *Eur J Hum Genet* 2008; **16**: 913–20.
- 43 Bowen DJ, Keeney S. Unleashing the long-distance PCR for detection of the intron 22 inversion of the factor VIII gene in severe haemophilia A. *J Thromb Haemost* 2003; **89**: 201–2.
- 44 Rossetti LC, Radic CP, Larripa IB, de Brasi CD. Genotyping the haemophilia inversion hotspot by use of inverse PCR. *Clin Chem* 2005; **51**: 1154–8.
- 45 WHO International Standard. 1st International Genetic Reference Panel for Haemophilia A, Intron 22 Inversion, Human gDNA. NIBSC code: 08/160. Instructions for use. Available at: <http://www.nibsc.ac.uk/documents/ifu/08-160.pdf>. Accessed 5 January 2009.