The UK National External Quality Assessment Scheme (UK NEQAS) for molecular genetic testing in haemophilia

David J. Perry¹, Anne Goodeve², Marian Hill³, Ian Jennings⁴, Steve Kitchen⁵, Isobel Walker⁶ and UK NEQAS for Blood Coagulation

¹Department of Haematology, Addenbrookes Hospital, Cambridge, UK; ²Division of Molecular and Genetic Medicine, Royal Hallamshire Hospital, Sheffield, UK; ³University Hospital, Queens Medical Centre, Nottingham, UK; ⁴UK NEQAS for Blood Coagulation, Sheffield, UK; ⁵Department of Coagulation, Royal Hallamshire Hospital, Sheffield, UK; ⁶Department of Haematology, Glasgow Royal Infirmary, Glasgow, UK

Summary

Molecular genetic analysis of families with haemophilia and other inherited bleeding disorders is now a common laboratory investigation. In contrast to phenotypic testing in which strict quality control is adhered to, in haemophilia molecular genetic testing there has been a lack of any external quality assurance schemes. In 1998 the UK National External Quality Assessment Scheme (UK NEQAS) established a pilot quality assurance scheme for molecular genetic testing in haemophilia. Results from three initial surveys highlighted problems with the quality of samples when used to screen for the intron 22 inversion within the F8 gene. The scheme was re-launched in 2003, and since that time there have been five exercises involving whole blood or immortalised cell line DNA. The results together with an overall summary of the exercise are subsequently returned to participants. Exercises to date have focused exclusively on haemophilia A and QA, material has included screening for the intron 1 and intron 22 inversions as well as sequence analysis. A paper exercise circulated in 2003 highlighted problems with the format of reports and, following feedback to participants, only a single error has been made in the subsequent four exercises. Participating laboratories now receive QA material every six months. Immortalised cell line material was introduced in 2005 and was shown to perform well. This will allow expansion of the scheme and a reduction in the dependence on blood donation.

Keywords

Quality assurance, haemophilia, molecular genetics, mutational analysis

Introduction

Genetic testing is amongst the most rapidly expanding fields in laboratory medicine, and the last 15 years we have seen a dramatic increase in requests for molecular analysis in haematology and in particular in haemostasis. This expansion has been driven in part by developments in laboratory methods which permit the rapid identification of genetic mutations and in part by the identification of novel genetic risk factors for disease, e.g. the factor V Leiden mutation. In the investigation and management of families with haemophilia, mutation analysis is now the method of choice for the clarification of carrier status in ‘at-risk’ women and for prenatal diagnosis, with linkage analysis being reserved almost exclusively for those small numbers of families in whom an underlying mutation cannot be identified.

Proficiency testing in haemostasis is a requirement for laboratory certification in the UK, although most research laboratories do not have such certification. In contrast to phenotypic data, the results of genotypic assays are unequivocal with no borderline values, and accordingly there is general acceptance of the correctness of such data by referring physicians. However, a number of studies have shown that mutation detection in common with any analytical tests has an intrinsic error rate (1–5).

In the European Union (EU), quality assurance within genetic testing laboratories is guided by a number of external quality assurance programmes (6, 7) which provide laboratories with samples for testing and subsequently with analyses of their individual and overall results. The results of these tests together with an overall summary are fed back to participating laboratories. Currently there is no quality assurance scheme for haemophilia molecular genetics in the EU. This paper summarises the development, implementation and the results of a quality assurance programme for haemophilia genetics in the UK.
Design of the external quality assurance scheme

The UK NEQAS (National External Quality Assessment Schemes) in haemophilia genetics was designed to address two important aspects of genetic testing. Firstly, the correct identification and interpretation of genetic mutations and secondly, the reporting of results. The correct interpretation of genotypic data in the light of the clinical history and the reason for the tests is fundamental to genetic studies. Furthermore, the report must be interpretable by clinicians who may not be clinical geneticists.

In the UK prior to the introduction of heat treatment of clotting factor concentrates in the mid-1980s, almost all severely affected patients with haemophilia A who were exposed to clotting factor concentrates were infected with hepatitis C (8) and 90% were infected with HIV (9). As a consequence, in many families the affected index case may have died and access to genetic material is no longer possible. It is, therefore, essential that no errors should be made in genetic studies.

In 1998 UK NEQAS (Blood Coagulation) established a pilot scheme for the detection of the intron 22 factor VIII gene inversion with additional exercises in 1999 and 2000. The results of these preliminary exercises highlighted problems with the quality of the samples when used for intron 22 analyses. During the following three years no exercises took place. However, the need for an EQA scheme in haemophilia genetics was seen as important both by the United Kingdom Haemophilia Centre Directors Organisation (UKHCDO) and by UK NEQAS (Blood Coagulation.) In 2003 a Special Advisory Group (SAG) on Haemophilia Molecular Genetics for UK NEQAS (Blood Coagulation) was established with the remit of developing a robust QA scheme for molecular genetics in the UK and which could be expanded internationally. Between 2003 and 2006 a further five exercises have been undertaken (Table 1), the most recent of which (exercise 8) was circulated in May 2006. Samples are distributed by UK NEQAS and returned evaluated by the SAG.

The intron 22 inversion within the factor VIII (F8) gene accounts for approximately 42% of cases of severe haemophilia A (10). In 1998 most laboratories in the UK were screening for the presence or absence of the intron 22 inversion by Southern-blot analysis but by 2005 almost all (8 of 10 laboratories) had switched to using a long-range PCR technique (11) with only a single laboratory utilising Southern-blot analysis and one laboratory inverse PCR (12). Screening for the intron 22 inversion by long-range PCR is probably the most challenging laboratory technique performed in the molecular characterisation of families with haemophilia A, due primarily to the large DNA fragment sizes (10–12 kb). However, as the intron 22 inversion is the most commonly occurring mutation in patients with severe haemophilia A, it is the first-line screening test performed in the investigation of such families. Mutation analysis in haemophilia A also includes screening for the less frequent intron 1 inversion (13) which constituted exercise 5; and sequence analysis which formed the basis of exercise 6.

Historically the size and complexity of the F8 gene (186kb/26 exons) led many laboratories to adopt some form of mutational screening technique, sequencing only those regions of the gene in which a mutation was suspected. However, today high-throughput relatively inexpensive sequencing technology has led many laboratories to abandon a screening approach and rely directly upon sequencing the coding regions, intron-exon boundaries and the 5' and 3' untranslated regions.

Table 1 summarises the seven exercises which have been fully evaluated to date. Mutations were chosen to reflect the routine workload in molecular genetic laboratories. Figure 1 shows the paper exercise circulated as exercise 4. All exercises provide laboratories with sufficient information to correctly identify the

<table>
<thead>
<tr>
<th>Exercise</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Samples</td>
<td>DNA x 3</td>
<td>WB x 3</td>
<td>WB x 2</td>
<td>Paper exercise</td>
<td>WB x 3</td>
<td>WB x 3</td>
<td>WB</td>
</tr>
<tr>
<td>Number of labs circulated</td>
<td>13</td>
<td>12</td>
<td>17</td>
<td>15</td>
<td>9</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Number of labs returning results</td>
<td>10 returned results</td>
<td>6 labs correct results</td>
<td>9 labs returned results</td>
<td>8 labs returned reports</td>
<td>8 labs returned reports</td>
<td>8 labs returned reports</td>
<td>10 labs returned results</td>
</tr>
<tr>
<td></td>
<td>3 produced no results – poor quality DNA</td>
<td>2 labs had problems with 1 sample (poor quality DNA)</td>
<td>2 labs correct results</td>
<td>4 of these labs failed</td>
<td>8 labs correctly identified the presence of absence of the mutation</td>
<td>8 labs correctly identified the presence of absence of the mutation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 labs – no results – poor quality DNA</td>
<td>7 labs reported linkage results in a variety of formats</td>
<td>7 labs did not return a report</td>
<td>1 lab had – difficulty in isolating DNA</td>
<td>1 lab incorrectly numbered the mutation</td>
<td>1 lab – difficulty in isolating DNA</td>
<td></td>
</tr>
<tr>
<td>Request</td>
<td>Intron 22 analysis</td>
<td>Intron 22 analysis</td>
<td>Intron 22 analysis + polymorphism analysis</td>
<td>Construct reports based upon standard in-house format</td>
<td>Intron 1 analysis</td>
<td>Sequence analysis: missense mutation in exon 14</td>
<td>Intron 22 inversion</td>
</tr>
</tbody>
</table>

SHA – severe haemophilia A (VIII:C < 1Iu/dl), MHA – mild haemophilia A (VIII:C > 31Iu/dl), WB – whole blood.
sample using two independent means (name and hospital number or date or birth), the diagnosis, the clinical question posed and any relevant pedigree and family phenotypic data. In addition, any genetic data that may already be available is provided together with what test(s) are requested. Any relevant information on nucleotide numbering is included to allow laboratories to correctly define and report a mutation. Exercises generally comprise two or three samples representing a family to be analysed.

Genotyping

In the routine diagnostic genetic laboratory, identification of mutations in the F8 gene involves screening and/or sequencing of the entire gene. However, to minimise the amount of work required for EQA exercises, laboratories are asked to screen for specific mutations (intron 22 or intron 1) or to sequence a defined part of the F8 gene.

In the 1998–2000 exercises, laboratories were asked to undertake only intron 22 inversion analyses and in the case of exercise 3, in addition to perform polymorphism (linkage) analysis. Lineage analysis was in common use at this time as routine mutational analysis was unrealistic for all but a small number of patients with inherited bleeding disorders. The major problem encountered by a number of laboratories was the quality of the DNA circulated or isolated by them from the whole blood samples. This in part reflected the difficulties with intron 22 analyses by Southern-blot analysis or long-range PCR six to seven years ago. Subsequent studies in 2004–2005 have not identified the quality of DNA/whole blood samples to be a major problem for the majority of laboratories. In exercises 5–7, all the mutations were correctly identified and the only error occurred in exercise 6 in which one laboratory reversed the nucleotide numbering of the mutation although correctly identified the 2-bp deletion of nucleotides CT in a repeated sequence between nucleotides 3414–3417 described as c.3416_3417delCT/p.Ser1120Trp fs.

Laboratory reports and scoring of reports

A fundamental part of any genetic investigation is the generation of an accurate and informative report. Of the three exercises circulated in 1998–2000 (Table 1), all of which involved screening for the presence or absence of the intron 22 inversion, scoring of laboratory interpretative reports was not undertaken. The introduction of a formalised template for scoring reports was introduced in 2003. The template (Fig. 2) was based upon the recommendations of the UK Clinical Molecular Genetics Society (CMGS) best practice guidelines on report writing (14) and was used to score three areas of the report: clerical accuracy, genotyping and interpretation.

Table 1

<table>
<thead>
<tr>
<th>Scored exercise</th>
<th>Family 1</th>
<th>Family 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Referring Hospital</td>
<td>St Elsewhere</td>
<td>County General Hospital</td>
</tr>
<tr>
<td>Referring consultant</td>
<td>Dr AN Other</td>
<td>Dr JS Bach</td>
</tr>
<tr>
<td>Patient Name</td>
<td>Max Headroom</td>
<td>Anna Bolic</td>
</tr>
<tr>
<td>Hospital Number</td>
<td>1123453</td>
<td>2456783</td>
</tr>
<tr>
<td>DOB</td>
<td>1/4/80</td>
<td>25/12/78</td>
</tr>
<tr>
<td>Diagnosis</td>
<td>Haemophilia A</td>
<td>?? Haemophilia A carrier</td>
</tr>
<tr>
<td>Other information</td>
<td>Adopted</td>
<td>Brother severe haemophilia A - FVIII:C&lt;2u/dl</td>
</tr>
<tr>
<td>Lab number</td>
<td>20303-1</td>
<td>20303-2</td>
</tr>
</tbody>
</table>

| Mutation data | C→T substitution at nucleotide 1834 in the FVIII gene 1,2 | Pattern consistent with heterozygosity for a distal FVIII gene inversion |

1Nucleotide numbering: +1 represents the first nucleotide of the signal peptide
2Amino acids numbered after scheme of Vehar et al. (1984) i.e. +1 is the first amino acid of the mature protein. The 19 signal peptide residues are numbered negatively.
3The entire coding region of the FVIII gene was sequenced including splice sites and the 5’ and 3’ UTR regions.

Figure 1: Exercise 4 (paper exercise).

The two categories — clerical accuracy and genotyping — were scored out of 35 each, whilst interpretation was scored out of 30. The maximum score was, therefore, 100. In each category information was considered to be either ‘essential’ or ‘recommended’, and a ‘fail’ in any category resulted in a laboratory failing that particular exercise. Laboratory identification numbers were assigned to each laboratory so that the scoring of each assessment exercise was anonymous. However, ID numbers were the same for each laboratory in all exercises allowing the rapid identification of poorly performing laboratories. Laboratories were scored independently by three individuals comprising the SAG with extensive experience in the field and a consensus subsequently reached. The scored template together with a summary document was returned to individual laboratories. Any laboratory that failed an exercise received a letter from the NEQAS Director (Blood Coagulation) with the offer of assistance.

In addition to the CMGS best practice guidelines for report writing (14), the UKHCO guidelines on the provision of genetics services highlight important aspects of the laboratory report (15). In practice, report formats differed widely between laboratories and whilst NEQAS was not prescriptive as to how reports should be structured a number of areas were highlighted. These included:
Reports should state the reason for carrying out the investigations.
- The severity of the disorder and any phenotypic data should be stated.
- The sample date should be included on all reports.
- Reports wherever possible should be condensed to fit onto a single page of A4 paper. If they extend onto two pages the report must state ‘page 1 of 2 etc’ on the footer.
- Reports should indicate how an analysis was performed and reference the technique if appropriate.
- Reports should maintain patient confidentiality by limiting identifying data on other family members.
- The clinical question should be answered unambiguously.
- Reports should highlight that mutational data could be used, for example, to establish carrier status in ‘at risk’ females.
- Previously reported mutations, e.g. on the HAMSteRS database (see http://europium.csc.mrc.ac.uk), should be indicated along with concordance of disease severity with the individual analysed. Novel mutations should include an indication of why they are considered pathogenic and how the possibility that they could represent polymorphic variation was excluded.

In the paper exercise (exercise 4) circulated in 2003, four laboratories failed on the basis of their reports. Reasons for failure included incorrect reporting of the mutation, failure to answer the clinical question(s) that were being asked and inadequate interpretation of the data. In the three subsequent exercises (5–7) circulated in 2004–2005, only a single laboratory failed in any of these exercises.

Mutation nomenclature
One of the remits of the advisory group was to encourage laboratories to use a uniform method for the reporting of mutations. In common with the CMGS, it was recommended that laboratories should use the system proposed by the Human Gene Variation Society (HGVS) (16). The adoption of the HGVS system requires all sequence variations to be in relation to a reference sequence (either a genomic or a coding DNA reference sequence) and the first nucleotide ‘A’ of the ATG-translation initiation codon to be numbered as +1 with the protein sequence representing the primary translation product, not a processed mature protein, and therefore includes signal peptide sequence data. For some proteins in which historically the first amino acid of the mature protein has been designated as +1 and the amino acids of the signal peptide numbered as –1, –2 etc, a renumbering of the protein is required. This makes reference to previously described mutations difficult unless the method of mutation nomenclature is given together with a reference for the gene/protein sequence. The HGVS system has evolved during the course of the NEQAS QA scheme and, whilst at present we have not penalised laboratories for not conforming to this format, its use is something that the EMQN and CMGS strongly advocate and which we have encouraged all participants in the scheme to adopt. In the context of an international QA scheme for haemophilia genetics it is clearly essential that participating laboratories adhere to a common universally agreed format for mutation nomenclature. Similarly, laboratories are encouraged to adopt a uniform format for the reporting of haplotype studies if these are undertaken in families in which a causative mutation cannot be identified.

Sample types
Of the seven exercises that have been evaluated, one was a paper exercise (exercise 4), one involved the circulation of DNA (exercise 1), and the remaining five involved the distribution of citrated whole blood samples. Ethical approval for all aspects of the EQA scheme and informed consent (where appropriate) was

![Figure 2: Formalised template for scoring reports.](http://europium.csc.mrc.ac.uk)
obtained. Samples were dispatched by post to participating laboratories and six weeks from receipt of samples was allowed for centres to undertake relevant investigations, construct a report and return the report to NEQAS.

The use of whole blood samples is more representative of the laboratory investigation of a haemophilic family although their use is in distinct contrast to most genetic QA schemes, which involve the use of DNA obtained from immortalised cell lines. Previous studies (unpublished) have shown that whole blood samples distributed in the UK by first-class mail should yield sufficient good-quality DNA for analysis, even when delays of several days occur. Back-up samples can be held at 4°C, and posted out if and when required.

As part of exercise 7 and to establish whether immortalised cell line material could be used as suitable material for haemophilia QA schemes, laboratories were provided with DNA from three cell lines for intron 22 inversion analyses (Table 2). Feedback from participants indicated some variation in the success of lyophilised DNA although this appeared to be independent of the technique and is more likely, therefore, to represent laboratory practice in sample reconstitution. Liquid DNA samples appeared, in general, to generate more consistent results but may not be suitable for long-term storage. The current exercise (exercise 8) circulated in May 2006 involves exclusively cell-line DNA. Immortalised cell-line material is the only material that can be used to extend the scheme internationally without prohibitively expensive transit conditions and therefore, to expand the number of participants. The use of such material also removes dependence upon patient availability for sample distribution.

Conclusion

The aim of EQA schemes is to highlight problems and deficiencies in laboratory procedures. Overall, the introduction of the scheme appears to have led to a more uniform inclusion of information into reports plus a more standardised use of mutation nomenclature. In the UK, laboratories undertaking genetic studies in patients with inherited bleeding disorders are obliged to participate in the QA scheme and it is a requirement for membership of the UKHCO Haemophilia Laboratory Network and for accreditation through CPA (Clinical Pathology Accreditation (UK) Ltd.).

To date the NEQAS Haemophilia Genetics QA scheme has focused exclusively on haemophilia A, providing two exercises per annum for its participants. It is envisaged that the scheme will be expanded into von Willebrand disease (VWD) and haemophilia B in 2006/7. It is probable that laboratories that perform well in a genetic QA scheme for haemophilia A will perform equally well for similar studies in haemophilia B. However, this may not be the case for VWD where the interpretation of the results is more complex. It is probable that any QA scheme for VWD molecular genetics will focus, at least initially on type 2 VWD as few laboratories are involved in routine molecular studies on type 1 VWD.

Acknowledgements

We are grateful to members of the UKHCO Haemophilia Laboratory Network for their invaluable feedback on the development and implementation of the NEQAS QA scheme. We are grateful to Elaine Gray and Paul Metcalf at the National Institute of Biological Sciences and Controls (NIBSC) for the preparation and subsequent isolation of DNA from immortalised cell lines.

Table 2: Haemophilia molecular genetics QA scheme: immortalised cell lines.

<table>
<thead>
<tr>
<th>Exercise</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Survey Year</strong></td>
<td>2005</td>
<td>2006</td>
</tr>
<tr>
<td><strong>Samples</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Lyophilised DNA:</td>
<td>Normal male</td>
<td></td>
</tr>
<tr>
<td>2. Liquid DNA:</td>
<td>Normal male</td>
<td></td>
</tr>
<tr>
<td>3. Liquid DNA: Haemophilia with the intron 22 inversion</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Request**

- Intron 22 analysis
- Sequence analysis

References