ORIGINAL ARTICLE

External quality assurance of molecular analysis of haemochromatosis gene mutations

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Background: The Royal College of Pathologists of Australasia Quality Assurance Programs has conducted an external quality assurance programme for the testing of the haemochromatosis gene (*HFE*) mutations *C282Y* and *H63D*.

Methods: A total of 10 surveys have been undertaken over a period of 6 years from 2000 to 2005.

Results: Of the 3016 responses received, the overall success rate was found to be 99.47% (3000/3016). A total of 16 errors were found, 6 for *C282Y* and 10 for *H63D*. Only one sample was associated with more than one error, in which 2 of 23 respondents classified a normal sample as heterozygotic for *H63D*. Overall performance was observed to vary minimally between surveys, from a low of 91.3% correct (21/23 responses) for a normal sample to 100% correct in most (85/100) samples. Of the 10 complete surveys, four returned a 0% error rate. In one survey in 2004, seven incorrect responses were returned by one laboratory, all of which were secondary to transcriptional errors. Overall success rates per assay were 99.61% (1532/1538) for *C282Y* and 99.32% (1468/1478) for *H63D*. Over a period of 6 years from 2000 to 2005, the proportion of respondents using polymerase chain reaction (PCR) and restriction enzyme analysis fell from 85% to around 30%, whereas the proportion of laboratories using real-time PCR rose from 5% to around 55%, as indicated by the questionnaire surveys of methods used by participants.

Discussion: Encouraging levels of testing proficiency for two common genetic mutations are indicated by these data, but they also confirm the need for participation of molecular diagnostic laboratories in external quality assurance programmes to ensure the ongoing provision of high-quality genetic testing services.

Hereditary haemochromatosis is an autosomal recessive disorder causing iron overload that is associated with mutation in the *HFE* gene. In most cases, the mutation is a single-base change that results in the substitution of tyrosine for cysteine at position 282 (*C282Y*) of the *HFE* protein.¹ Homozygosity for the *C282Y* mutation is present in approximately 8 of every 1000 people of northern European descent;² however, there is an apparently low clinical penetrance such that the genotype may not fully explain the presenting symptoms for many patients.³⁻⁴ Other mutations in *HFE* have also been described. One of these variants results from a substitution of an aspartic acid for a histidine at amino acid 63 (*H63D*) and has an allele frequency of about 15% among northern Europeans.⁵⁻⁶ The clinical effects of this mutation seem to be limited, although 1–2% of people with compound heterozygosity for *C282Y* and *H63D* are predisposed to developing an iron-loading syndrome.⁷ Over the past few years, the number of laboratories carrying out these molecular genetic assays as well as the number of requests for tests has dramatically increased.

The detection of these common *HFE* mutations by molecular genetic analysis has the advantage of being able to detect a mutation without relying on phenotypic parameters. Unlike phenotypic testing, the genotypes are unequivocal, with no borderline values. Accordingly, the reliability of the result is totally accepted by referring doctors. As the results of molecular genetic testing for inherited disorders may have important clinical and familial implications, it is essential that laboratories undertake stringent internal quality measures in identifying critical steps in the pre-analytical phase, the analytical phase and the post-analytical phase of genetic testing.⁸

There are a few external quality assurance programmes for DNA testing of disorders such as haemochromatosis. These include the US College of Pathologists (CAP), the European

Molecular Genetics Quality Network (EMQN) and the UK National External Quality Assessment Scheme (NEQAS). Such programmes have indicated that, although concordance is relatively high, analytical errors and handling and mislabelling errors do occur, indicating the need for ongoing participation of molecular laboratories in external quality assurance assessment.⁹ The Royal College of Pathologists of Australasia Quality Assurance Programs (RCPA QAP) recently introduced modules for the testing of the two common *HFE* mutations. We report the results of 10 surveys, on 50 different DNA samples, that were undertaken over a period of 6 years from 2000 to 2005.

SUBJECTS AND METHODS

In 2000, the RCPA QAP introduced a quality assurance module for the testing of the hereditary *HFE* mutations, *C282Y* and *H63D*. Since then, 3300 aliquots of purified DNA from 50 different blood samples have been despatched in 10 separate surveys. Currently, 37 laboratories are enrolled in this programme, including those in Australia, New Zealand, South Africa, Southeast Asia, India and Europe. Participating centres include university teaching hospital service laboratories, as well as research and private laboratories.

At the central RCPA QAP laboratory, DNA was extracted from whole blood samples collected in ethylenediamine tetraacetic acid (no heparin) that were derived from people of known genotypes for each of the two *HFE* mutations. Samples were then diluted in water to a final concentration of 100 µg/ml and checked for homogeneity by random sample selection for confirmation of genotypes by DNA amplification and restriction enzyme digestion.

Abbreviations: *HFE*, haemochromatosis gene; PCR, polymerase chain reaction; RCPA QAP, The Royal College of Pathologists of Australasia Quality Assurance Programs

Subsequently, 0.5 µg (minimum) or 1.0 µg of DNA from each sample was aliquoted into Eppendorf tubes (sufficient to repeat assays on at least two or three occasions), and then distributed to the participating laboratories by surface post. Five separate DNA samples were despatched at ambient temperature to each participant in each survey and were each tested for both *HFE* point mutations.

In 2000 and 2005, questionnaire surveys were also conducted, in which information on the methods used to detect *HFE* mutations, together with the cited literature reference, was sought from each participating laboratory.

RESULTS

Laboratories were unable to amplify 0.42% (14/3300) of the samples and results were not received for 8.2% (270/3300) of the samples. The category of results not received was derived from laboratories that returned entire surveys for reasons including temporary suspension of molecular testing, test procedure under development and logistical or staffing issues. Of the remaining 3016 responses received, the overall success rate was 99.47% (3000/3016). Incorrect responses included those caused by both laboratory scientific as well as typographical errors (table 1). A total of 16 errors were observed: 6 for *C282Y* and 10 for *H63D*. Of the 16 confirmed errors, three occurred in a survey in 2000, whereas in a survey in 2004 one respondent returned seven incorrect responses, all of which were transcriptional errors. Only one sample was associated with more than one error—that is, 2 of 23 respondents classified a normal sample as heterozygotic for *H63D*.

Overall performance varied slightly between surveys, from a low of 91.3% correct (21/23 responses) for a normal sample to 100% correct in most (85/100) samples (table 1). That is, most (85/100) samples returned a 0% error rate (ie, 100% correct response), whereas 4 of the 10 complete surveys from 2000 to 2002 returned a 0% error rate. Overall success rates per assay were 99.61% (1532/1538) for *C282Y* and 99.32% (1468/1478) for *H63D*. Table 2 summarises the key findings from this study.

Table 3 summarises the results of the questionnaire survey of methods used in 2000 and 2005. The results indicate that, over time, the proportion of laboratories using polymerase chain reaction (PCR) and restriction enzyme analysis fell from 85% to 28–34%, whereas the proportion of laboratories using real-time PCR rose from 5% to 52–59%. In addition, laboratories cited as many as 29 different references for their particular analytical method.

DISCUSSION

This is the first major report of results from a large quality assurance programme for the molecular testing of *HFE* mutations. It indicates encouraging levels of testing proficiency across more than 30 participating laboratories in several countries, as the overall accuracy of detection of *HFE* mutations is 99.47%. Given the number of exercises conducted and the large sample size in the RCPA QAP surveys, the results are probably a true reflection of laboratory practices in which tests are conducted both in large university teaching hospital service laboratories and in research laboratories and private facilities. Overall, relatively few errors were confirmed, and these could be as facile as an inadvertent switch in the provided samples, handling or transcription errors. Irrespective of the cause, these errors should not occur and must be included in the analysis of reported results from participating laboratories.

The questionnaire surveys indicate that, over time, there has been a substantial shift in technology and methods used for detection of the two *HFE* mutations. That is, over a period of 6 years from 2000 to 2005, the proportion of laboratories using PCR and restriction enzyme analysis fell from 85% to around 30%, whereas the proportion of laboratories using real-time PCR rose from 5% to around 55%. Similarly, we observed a corresponding change in the use of PCR and restriction enzyme digestion in favour of real-time PCR in detecting the thrombophilia gene mutations (unpublished observations, RCPA QAP). Indeed, for many laboratories, particularly those servicing the private sector, real-time PCR offers substantial advantages, including the provision of

Table 1 Summary of data from The Royal College of Pathologists of Australasia Quality Assurance Programs HH DNA testing

Survey year	2000a	2000b	2001	2002a	2002b	2003a	2003b	2004a	2004b	2005a	
(A) Cys282Tyr											
Number of laboratories	27	31	31	35	34	32	33	37	37	36	
Details of samples (n)											Overall
All	5	5	5	5	5	5	5	5	5	5	50
Normal	2	1	1	1	1	1	2	3	3	2	17
Heterozygotic	2	3	3	3	4	3	2	1	1	2	24
Homozygotic	1	1	1	1	0	1	1	1	1	1	9
Errors	0	0	0	0	0	0	1	0	4	1	6
Error rate (%)											Average
All	0	0	0	0	0	0	0.65	0	2.38	0.57	0.39
Normal	0	0	0	0	0	0	0	0	3.03	1.43	0.75
Heterozygotic	0	0	0	0	0	0	0	0	0	0	0
Homozygotic	0	0	0	0	0	0	3.20	0	2.86	0	0.65
(B) His63Asp											
Number of laboratories	27	32	30	34	33	31	32	36	36	36	
Details of samples (n)											Overall
All	5	5	5	5	5	5	5	5	5	5	50
Normal	3	2	3	2	2	2	2	2	2	2	22
Heterozygotic	1	2	1	2	2	2	2	1	2	2	17
Homozygotic	1	1	1	1	1	1	1	2	1	1	11
Errors	3	0	0	0	0	1	0	2	4	0	10
Error rate (%)											Average
All	2.63	0	0	0	0	0.69	0	1.27	2.38	0	0.68
Normal	8.70	0	0	0	0	0	0	0	3.03	0	0.62
Heterozygotic	4.55	0	0	0	0	3.57	0	0	1.47	0	0.58
Homozygotic	0	0	0	0	0	0	0	3.23	2.94	0	0.91

Table 2 Key findings from The Royal College of Pathologists of Australasia Quality Assurance Programs haemochromatosis gene surveys

Error rates per sample ranged from 0% to a high of 8.7% for a normal sample.
 Most (85/100) samples returned a 0% error rate (ie, 100% correct response).
 Overall success rate was 99.47% (3000 correct of 3016 returns).
 Overall success rates per assay were 99.61% (1532/1538) for *C282Y* and 99.32% (1468/1478) for *H63D*.
 Of the 10 surveys, 4 returned a 0% error rate.
 A total of 16 errors were observed, 6 for *C282Y* and 10 for *H63D*.
 One participant was responsible for seven errors in a survey in 2004.
 One sample was associated with more than one error—that is, 2 of 23 classified a normal sample as heterozygotic.

kit-based assays, large-volume sampling and rapid turn-around times.

Despite the obvious shift in technologies, there has been no obvious commensurate change in participant performance. In this regard, it is notable that one of the newly subscribing participants returned seven incorrect responses in one recent survey alone, all of which were the result of transcriptional errors. Hence, it is difficult to draw any definitive correlation of performance with methods, as not only is the overall error rate very low but most errors seem related to sample handling and transcription.

The overall performances of participants in the haemochromatosis surveys seem to be slightly more favourable than those found for other similar external quality assurance surveys on thrombophilia gene mutations, in which errors in diagnosis average between 1% and 3%.^{10–13} In our own surveys on thrombophilia gene mutations, among the 3799 responses received, the overall success rate was 98.6%, including rates of 98.1% for factor V Leiden, 98.8% for prothrombin *G20210A* and 99.3% for the *MTHFR C677T* mutation.¹³ The cause of the differences in error rates between the quality assurance programmes for thrombophilia and *HFE* gene mutations is not immediately apparent, and again cannot be readily correlated with changes in methods. Nevertheless, among responding laboratories in the thrombophilia programme, 3 of the 39 laboratories were responsible for 46% (24/52) of all errors in the first few years of the programme.¹³ Moreover, in the past 3 years, the error rates in detecting the three thrombophilia gene mutations seem to have fallen to a similarly low level of <1% (unpublished observations). Hence, one of the possible explanations for the

Take-home messages

- External quality assessment of molecular testing of two haemochromatosis gene mutations indicates encouraging levels of testing performance across more than 30 laboratories in a number of countries.
- The overall accuracy of detection of the *C282Y* and *H63D* point mutations is 99.47%.
- The relatively few errors were related to handling and transcription, with a minority being caused by technical issues.
- Over a 6-year period from 2000 to 2005, the proportion of laboratories utilising PCR and restriction enzyme analysis fell from 85% to around 30%, coincident with a rise in the use of real-time PCR from 5% to 55%.

difference in performance between the two quality assurance programmes seems to be related to the commencement of the thrombophilia programme 2 years before that of the haemochromatosis programme.

For these exercises, the RCPA QAP has not dealt with the issue of DNA extraction and its effect on the provision of accurate genotyping of the *HFE* and other mutations. Furthermore, surveys on individual PCR cycling temperatures and cycling number have not been possible. Accordingly, comprehensive data on correlation of response results with methods and thermal cycling characteristics are not available. Nevertheless, an indication of the methodological diversity is evident in the fact that responding laboratories cited as many as 29 different references for their detection methods.

Ideally, stable and reliable genetic reference materials such as cell lines ought to be available for comprehensive testing proficiency exercises rather than relying on patient DNA samples. Indeed, most laboratories use blood or extracted DNA samples from patients with known genotypes as their in-assay controls, and minimum practice standards indicate that these should be run with each batch. As the spectrum of genotype controls may not be universally applied, it is desirable that an international reference panel of DNA samples carrying these mutations is established. Accordingly, the National Institute for Biological Standards and Control recently established the first WHO Genetic Reference Panel for factor V Leiden by using immortalised cell lines produced by Epstein–Barr virus transformation of blood from donors who were known to carry the wild-type, homozygote and heterozygote genotypes for factor V Leiden.¹⁴ Similar plans are presently under way to include samples for prothrombin *G20210A*, haemophilia A and B and the *HFE* mutations *C282Y* and *H63D*.

Table 3 Results of methodology surveys

Method	2000		2005	
	<i>C282Y</i> (n)	<i>H63D</i> (n)	<i>C282Y</i> (n)	<i>H63D</i> (n)
Real-time fluorescent resonance-energy transfer	1	1	12	11
Real-time Taqman	0	0	5	4
Restriction enzyme digest	17	17	8	10
Allele-specific oligonucleotides	1	1	3	2
Oligonucleotide ligation assay	1	1	0	0
Pyrosequencing	0	0	1	1
Single-strand conformational polymorphism	0	0	0	1
References cited	5	5	29	29

Overall, the results of the RCPA QAP external quality assurance exercises described in this report are encouraging, indicating a promising level of accuracy for the detection of two common single-point mutations. They are concordant with results of previous studies on three thrombophilia gene mutations (factor V Leiden, prothrombin *G20210A* and MTHFR thermolabile variant) that confirm the accuracy of most results obtained from most laboratories.^{10–13} Indeed, the overall performance on most surveys is generally very good for all analyses, whereas most errors can be traced to handling, labelling or transcription. These studies, however, still emphasise the need for ongoing rigorous attention to molecular laboratory procedures and practices.^{9–13} It is only through participation in large quality assurance programmes that high levels of proficiency testing of inherited genetic mutations can be maintained as part of the provision of high-quality diagnostic services.

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