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ARTICLE

Discrepancies in reporting the CAG repeat lengths for Huntington's disease

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Huntington's disease results from a CAG repeat expansion within the *Huntingtin* gene; this is measured routinely in diagnostic laboratories. The European Huntington's Disease Network REGISTRY project centrally measures CAG repeat lengths on fresh samples; these were compared with the original results from 121 laboratories across 15 countries. We report on 1326 duplicate results; a discrepancy in reporting the upper allele occurred in 51% of cases, this reduced to 13.3% and 9.7% when we applied acceptable measurement errors proposed by the American College of Medical Genetics and the Draft European Best Practice Guidelines, respectively. Duplicate results were available for 1250 lower alleles; discrepancies occurred in 40% of cases. Clinically significant discrepancies occurred in 4.0% of cases with a potential unexplained misdiagnosis rate of 0.3%. There was considerable variation in the discrepancy rate among 10 of the countries participating in this study. Out of 1326 samples, 348 were re-analysed by an accredited diagnostic laboratory, based in Germany, with concordance rates of 93% and 94% for the upper and lower alleles, respectively. This became 100% if the acceptable measurement errors were applied. The central laboratory correctly reported allele sizes for six standard reference samples, blind to the known result. Our study differs from external quality assessment (EQA) schemes in that these are duplicate results obtained from a large sample of patients across the whole diagnostic range. We strongly recommend that laboratories state an error rate for their measurement on the report, participate in EQA schemes and use reference materials regularly to adjust their own internal standards.
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INTRODUCTION

Huntington's disease (HD) is an autosomal dominant progressive neurodegenerative disorder with onset usually, but not exclusively, in adult life. In 1993, the causative mutation was identified as an unstable CAG repeat expansion in the first exon of the *Huntingtin (HTT)* gene.[1] This was rapidly introduced into clinical practice for diagnostic and predictive testing.[2] Guidelines for reporting genetic test results were established by the American College of Medical Genetics (ACMG) and the American Society of Human Genetics, which state: a CAG repeat length of less than 27 is unequivocally normal; a CAG repeat length of 27–35 is normal but there may be expansion into the pathological range in future generations; a CAG repeat length of 36–39 is an abnormal result but there may be reduced penetrance; a CAG repeat length of 40 or more is unequivocally abnormal.[3]

External quality assessment (EQA) schemes have developed to ensure the accuracy of laboratory reporting.[4] The model for these schemes is that validated aliquots of DNA samples from one or more patients with known genotypes are circulated to participating laboratories for analysis of the CAG repeat length; the reports generated are collated centrally and ideally, all participating laboratories should generate the same genotype results and reports with similar interpretations.

In this paper we present the results from a different model of assessing the accuracy of genotype reporting: a fresh blood sample was taken from a large sample of individuals participating in the European Huntington's Disease Network (EHDN) REGISTRY project; the DNA was extracted and CAG repeat length for both alleles was measured by a central laboratory and the result compared with that obtained from the local service laboratory.

MATERIALS AND METHODS

Patient Samples

The EHDN REGISTRY project[5] is a multinational observational study; more details are available at http://www.euro-hd.net/html/registry. The data recorded from participants includes the result of the CAG repeat length reported by the local service laboratory. Participants have an option of donating fresh blood samples, which are taken in acid citrate dextrose (ACD tubes Vacutainer, Becton Dickinson, Milan, Italy) and couriered to the central laboratory, BioRep, Milan.

Laboratory Analysis

DNA was extracted from whole blood using the salting-out procedure,[6] and the *HTT* gene CAG repeat length analysed by PCR amplification followed by capillary electrophoresis using the MegaBace Fragment Profiler Software from General Electric (Buckinghamshire, UK)[7,8] using the following primers:

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HD1: 5′-FAM-ccttcgagtccctcaagtccttc-3′
HD5: 5′-cggctgaggcagcagcggctgt-3′
Hu4: 5′-HEX-atggcgaccctggaaaagctgatgaa-3′
Hu5: 5′-ggcggtggcggctgttgctgctgctgctgc-3′

Each sample was amplified with two primer sets: Hu4/Hu5, which amplifies CAG repeat sequences only and not adjacent CCG polymorphic variant. CCG and CAG are amplified with primer set HD1/HD5, this allows the detection of heterozygous CAG/CCG repeats. The reported results gave the CAG repeat number only.

BioRep participated in the EQA schemes organised by the European Molecular Genetics Quality Network (EMQN) on alternate years and the results are always congruent.

A second, independent, accredited laboratory in Tübingen, Germany, duplicated CAG repeat analyses for a subset of 348 DNA samples using the same DNA extracted at BioRep and the same primer sets. The amplified DNA was separated using the Applied Biosystems (Carlsbad, CA, USA) capillary electrophoresis. From 2002, the Tübingen laboratory has participated in the national (BVDH; 2002–2007) and European (EMQN, starting 2008) quality measures. All quality measure samples have been genotyped without any results outside error limits (accuracy within ± 1 CAG for repeats below 42 CAG repeats; accuracy within ± 1 for large HD mutations). The Tübingen lab has had a consistent discrepancy of -1 CAG allele in the lower repeat (which has been accounted for and corrected in this data series). The results obtained from the two laboratories were compared: the Tübingen values were subtracted from those of BioRep's.

In total, 1326 fresh samples collected between January 2004 and June 2009 were available for analysis. Duplicate results were compared by subtracting the BioRep result from that obtained from the local service laboratory for both the upper and lower allele. The number of samples reported from individual laboratories varied widely; hence, we aggregated the results for 10 countries contributing more than 20 samples to the study. We then applied the acceptable measurement errors to the discrepancies as proposed by the ACMG:[9] these are: ± 1 for CAG repeat lengths ≤ 43; ± 2 for CAG repeat lengths between 44 and 50; ± 3 for CAG repeat lengths between 51 and 75; ± 4 for CAG repeat lengths > 75.

The European Best Practice Guidelines (BPG) for HD is still in draft form;[11] however, the suggested measurement errors in that document are: ± 1 for CAG repeat lengths ≤ 42 and ± 3 for CAG repeat lengths ≥ 43. We tested the effect of this standard on our data.

Use of Reference Materials

The US National Institute for Standards and Technology (NIST) developed standard reference materials for HD.[10] The standards consisted of CAG repeat lengths of: sample 1, 15 and 29; sample 2, 7 and 36; sample 3, 15 and 40; sample 4, 35 and 45; sample 5, 39 and 50 and sample 6, 17 and 75. BioRep analysed these samples blind to the known CAG repeat size.

Participation in EMQN EQA

The names of laboratories participating in the 2009 EMQN EQA could not be disclosed for reasons of anonymity; however, the list of 121 laboratories participating in this study was sent to the EMQN coordinator who reported on the number participating in the 2009 EQA.

RESULTS

Duplicate results for the upper allele were available for 1307 patients and 19 non-mutation carrying controls who had a family history of HD. There were 654 (49%) concordant results and 672 (51%) discrepancies, which are summarised in Table 1: 31% were discrepant by one CAG repeat, 12% were discrepant by two CAG repeats and 8% had a discrepancy of three or more CAG repeats. The discrepancies were in both directions with 370 (55%) showing an increase, and 302 (45%) showing a decrease in size when re-analysed by BioRep. The discrepancy rate fell to 13.3% when we applied the acceptable measurement errors proposed by the ACMG and 9.7% when we applied the proposed BPG measurement error. The local results came from 121 laboratories, of these, 45 (31%) participated in the 2009 EMQN EQA. It is not known how many laboratories participated in national quality assessment schemes. Discrepancies came from 86 laboratories (71.1%): of these, 49 laboratories (40.5%) had discrepancies outside the proposed ACMG measurement error and 41 (33.9%) were outside the proposed BPG measurement error.

The number of samples each laboratory contributed to the study varied considerably; 103 laboratories contributed less than 20 local results, whereas 4 laboratories contributed 50 or more local results. We did not wish to identify individual laboratories and we did not think it was fair to compare the measurement error of a laboratory contributing more than 50 results with ones contributing only one or two results, hence, we aggregated the local laboratories' results by country. The local laboratories were distributed across 15 countries; however, five each contributed less than 20 results to the study and were excluded from the analysis of discrepancy rate by country. Figure 1 shows the discrepancy rate for 10 countries, which represents 1276 duplicate samples (96.2% of the total) together with the effect of applying both the proposed ACMG- and Draft BPG-acceptable measurement errors. The results for country 1 suggest there is a consistent error in reporting the upper allele. There was a wide variation in the discrepancy rate by country even after allowing for acceptable measurement errors.

A discrepancy is clinically significant if it crosses a boundary at 35–36 or 39–40 CAG repeat lengths; this occurred in 52 (4%) patients. Results for the upper allele changed from the reduced to the full penetrance range in 36 cases, whereas in 11 cases they moved from the full to the reduced penetrance range. A potential misdiagnosis occurred in five (0.4%) cases, which changed at the critical 35–36 CAG repeat length boundary. In one case, this was because of a labelling error on the fresh sample sent to BioRep; this leaves four cases (0.3%) with an unexplained potential misdiagnosis.

Methods of measuring the CAG repeat length in local laboratories have developed since 1993; hence, we considered the effect of this on our data. Of the 672 discrepant samples, the year in which the local laboratory reported the result was known for 663/672 samples; 89 discrepancies occurred in the years 1993–1996, and 549 occurred in the period 2006–2009; these represent 66.3 and 46.6% of the total samples received for the respective time periods. Applying acceptable measurement errors from the ACMG for these two time periods gave discrepancy rates of 13.5% and 12.8%, respectively. Applying the Draft-proposed BPG measurement errors for the same periods gave discrepancy rates of 7.9% and 8.9%, respectively.

Duplicate results were available for the lower allele in 1250 cases, of which 757 (60.6%) were concordant. The 493 (39.4%) discordant results are summarised in Table 2. Following re-analysis by BioRep, 153 (31%) of the discrepancies were an increase in the CAG repeat size, and 340 (69%) were a decrease in CAG repeat size. Applying either the ACMG or the BPG measurement limits resulted in 160 (12.8%) cases being discordant. The two cases with a lower allele CAG repeat length of more than 36 were reported as having a result of 37 and 50 CAG repeats and 39 and 45 CAG repeats for the lower and upper allele, respectively, by both the local service laboratory and BioRep.

In the case of a discordant result, it is not possible to say whether the local laboratory or BioRep gave the correct CAG repeat size. We tested the reliability of BioRep in two ways. First, a subset of 348 DNA samples was analysed at a second laboratory in Tübingen. The results were concordant for the upper and lower alleles in 324 (93%) and 327 (94%) cases, respectively. The upper allele decreased in size by 1 CAG

Table 1 Direction and magnitude of discrepancies in reporting the upper allele in 1307 patients and 19 controls (local service laboratory result minus BioRep result)

		Discrepancy							
Repeats	*0*	*Increase of 1 CAG*	*Decrease of 1 CAG*	*Increase of 2 CAG*	*Decrease of 2 CAG*	*Increase of 3–5 CAG*	*Decrease of 3–5 CAG*	*> ± 5*	*Total*
<36	6	1	2	0	1	0	6	3	19
36–39	23	4	12	3	0	0	2	0	44
40–44	433	140	98	36	59	27	30	6	829
45–49	163	73	33	14	25	10	6	6	330
50–54	17	25	7	6	5	1	2	0	63
55+	12	8	6	9	0	2	4	0	41
Total	654	251	158	68	90	40	50	15	1326
% Of total (/1326)	49.3	18.9	11.9	5.1	6.8	3.0	3.8	1.1	

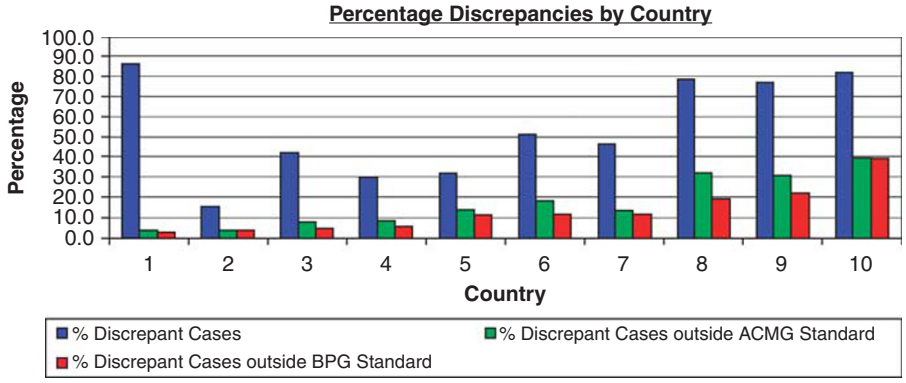

Figure 1 Summary of the percentage of discrepancies in reporting the upper allele for 1281 duplicate samples distributed across 10 European countries. The actual percentage is shown followed by the percentage after correcting for acceptable measurement errors proposed by both the ACMG and BPG, respectively.

Table 2 Direction and magnitude of discrepancies in reporting the lower allele for 1250 cases (local service laboratory result minus BioRep result)

		Discrepancy							
Repeats	*0*	*Increase of 1 CAG*	*Decrease of 1 CAG*	*Increase of 2 CAG*	*Decrease of 2 CAG*	*Increase of 3–5 CAG*	*Decrease of 3–5 CAG*	*> ± 5*	*Total*
<27	730	107	214	17	52	17	61	9	1207
27–35	25	8	4	0	0	2	0	2	41
36+	2	0	0	0	0	0	0	0	2
Total	757	115	218	17	52	19	61	11	1250
% Of total (/1250)	60.6	9.2	17.4	1.4	4.2	1.5	4.9	0.9	

repeat in 17 cases and increased by 1 CAG repeat in 7 cases at Tübingen. For the lower allele, 17 cases decreased and 4 cases increased in size by 1 CAG repeat at Tübingen. This represents complete concordance if either the ACMG- or BPG-proposed measurement errors are applied. In three cases, the discrepancy of 1 CAG repeat occurred in both the upper and lower allele. Second, BioRep was unaware of the known CAG repeat length for the six reference samples supplied by NIST; 11 alleles were reported correctly, but the 75 CAG standard was reported as 74, which is well within the margin of error for this allele size.

DISCUSSION

In a series of 1326 duplicate samples, we found a discrepancy in reporting the upper CAG repeat allele outside the ACMG- and BPG-proposed measurement errors in 13.3% and 9.7% of cases, respectively. The observation that the discrepancies were in both directions suggests that the result was not due to a consistent error at BioRep.

We considered the possibility that the explanation for our results is that the discrepancies occurred in earlier years; however, 41% of the samples in this study came from the period 2006–2009, with discrepancy rates outside the proposed ACMG and BPG acceptable

measurement errors of 12.8% and 8.9%, respectively, indicating that there is a current problem, which needs to be both recognised and addressed.

The purpose of this report is not to identify any specific laboratory, but rather to address the question of whether the problem is widespread or confined to just a few laboratories. The observation that the number of laboratories with discrepancies outside the proposed ACMG- and BPG-acceptable measurement errors is 49 and 41, respectively; in addition, the data from Figure 1 indicates that the problem occurs across Europe and is not confined to few countries. Country 1 had a significant number of samples discrepant by 1 CAG repeat, which is within acceptable measurement limits. The difference in measurement between two laboratories does not allow us to say which is the correct measurement, but the issue may well be resolved by more widespread use of standard reference materials.

There have been previous reports of discrepancies in the measurement of CAG repeat length. The 1999 report of the EQA scheme for HD, organised by the EMQN, asked laboratories to report on five samples with set measurement limits of ± 1 repeat for values up to 40, and ± 3 repeats for results of more than 40 repeats; 6.2% of results fell outside these limits and there was a potential misdiagnosis rate of 1.3%.[4] Although the methods adopted by an EQA and our study are not directly comparable, we note that follow-up data reported in 2009, show that after more than 10 years of EQA, based on three samples being circulated among laboratories, a potential misdiagnosis rate of 1–4% still occurred.[12]

In a study of reduced penetrance alleles, 200 samples were collected from centres reporting results in this range, but 24 (12%) had to be excluded because duplicate results from two reference laboratories showed that these had results of ≥ 40 repeats.[13] In the same study, a total of 238 duplicate results were available from the two reference centres; 10 (4.2%) differed by one CAG repeat in the reporting of the upper allele, and in 2 (0.84%) of these cases, the discrepancy was between 39 and 40 repeats.[13]

Our study was based on duplicate reporting of results for 1307 patients across the whole spectrum of CAG repeat length, and showed that discrepancies in measuring and reporting this are common; clinically significant discrepancies occurred in reporting the upper allele in 4.0% of the sample, with an unexplained diagnostic error rate in 0.3% in this large patient series. The fact that our study considered results from across the diagnostic range may explain why the unexplained misdiagnosis rate is less than that suggested from the EQA studies, which are more likely to concentrate on samples at clinically important boundaries.

As part of the standard operating procedure for our REGISTRY study, principal investigators receive a periodic printout of the results obtained from BioRep together with the patient's pseudonym so they are in a position to check a specific result. If a patient has a CAG repeat length well above 40 repeats and there is a discrepancy of one or more CAG repeats then this may not have any clinical consequence but, knowledge of the degree of discrepancy that can occur may be important for those undertaking research, based on CAG repeat length data pooled from multiple service laboratories.

We have neither identified the countries contributing samples nor have we sought to identify specific laboratories within countries; our aim is to draw attention to the wide variation, which exists across Europe and that it is not specific to one country. It is not possible to explain the cause of the variation in the results between laboratories from this study but one possibility could be the use of different electrophoretic matrices.[14] The data do not allow us to investigate all the possible causes of the discrepancies but, having identified that a problem exists, we wish to suggest ways of addressing the issues. Our result suggests that laboratories should quote an error rate in reporting the allele sizes; this error rate may increase as the allele size increases. There are no universally agreed acceptable measurement errors hence we recommend laboratories determine the error rate experimentally rather than choosing rates from a published recommendation. This will allow clinicians to be aware that the result is not an absolute but, as with all measurements, is subject to error. Discrepancies outside acceptable measurement limits continue to occur, despite the participation of laboratories in EQA schemes; use of standard reference materials to calibrate internal standards should result in a further reduction of the scatter in measurements.

Cases of clear misdiagnosis may be rare, but there may be more problems at the 39/40 boundary; the genetic counselling differs slightly depending on whether the result is in the reduced penetrance or unequivocally abnormal range. We strongly recommend that service laboratories should participate in EQA schemes, but, in addition, should use known reference materials regularly to adjust their own internal standards.

CONFLICT OF INTEREST

G Bernhard Landwehrmeyer received research grants from CHDI and honoraria from Siena Biotech for consultations as well as compensation for the conduct of clinical trials from Amarin, Neurosearch, Novartis and Medivation. The remaining authors declare no conflict of interest.

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APPENDIX

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