A comparison of methods for gene dosage analysis in HMSN type 1

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Abstract

A number of different approaches are used in diagnostic laboratories to detect the 1.5 Mb duplication at 17p11.2 seen in approximately 70% of patients with hereditary motor and sensory neuropathy type 1 (HMSN1). Here we compare the methods used in UK diagnostic laboratories to detect the duplication. Samples referred to participating centres for HMSN testing were collected, randomised, and distributed for testing. One hundred samples were examined using five different methods; each method was tested by two independent laboratories. Identical results were obtained from all laboratories for 44 samples. The remaining samples were classified as duplication positive or duplication negative on the basis of the same result by two or more methods. A total of 95 samples were classified by more than one method, two were withdrawn from the study as the same result was not obtained by two methods, and three are thought to have a duplication smaller than 1.5 Mb. Seven of 49 duplications were not detected by methods used to detect the common junction fragment and the use of microsatellites failed to yield a result in four of 95 samples. Sequence tagged site (STS) dosage analysis was found to be the most sensitive of the methods tested, although this method was found to be the most likely to require repeat analysis. Eight samples gave discordant results between the two laboratories testing by the same method. Upon retesting, reasons for the initial incorrect result included processing and typographical errors.

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Keywords: HMSN; methods; duplication; gene dosage

The hereditary motor and sensory neuropathies (HMSN) or Charcot-Marie-Tooth disease (CMT) are a clinically and genetically heterogeneous group of disorders. HMSN type 1 (HMSN1) (MIM 118220) is the most common with an incidence of about 1/2500.

Approximately 70% of HMSN1 patients have a tandem duplication of a 1.5 Mb region at 17p11.2 (HMSN1A).¹⁻³ The duplicated region includes the peripheral myelin protein gene-22 (*PMP-22*) which encodes a component of peripheral nerve myelin.⁴⁻⁷ Trisomic overexpression of *PMP-22* is thought to be the cause of the neuropathy in this group of patients. The involvement of *PMP-22* in HMSN pathogenesis is supported by the finding of point mutations in *PMP-22* in patients without a duplication.⁸ Two homologous, low copy number, repeat elements flank the repeated region and are designated distal or proximal CMT1A-REPs according to their position.² Duplication and the reciprocal deletion events are thought to arise during meiosis owing to non-sister chromatid exchange between misaligned CMT1A-REP elements.^{2,9} Although the CMT1A-REPs are approximately 24 kb in size, a hot spot for recombination exists between an *EcoRI* site unique to the distal CMT1A-REP and a *SacI* site unique to the proximal CMT1A-REP. In duplicated cases, the recombinant CMT1A-REP contains both sites producing a disease specific junction fragment of 3.2 kb. The recombination hot spot is further refined to a 1.7 kb region between the *EcoRI* site of the distal CMT1A-REP and a unique *NsiI* site on the proximal CMT1A-REP.¹⁰

The methods used in diagnostic laboratories to detect the 17p11.2 duplication include quantitative methods and methods to detect rearrangements. The aim of this study was to compare the methods used in UK diagnostic laboratories and to determine the sensitivity and reproducibility of each method.

Methods

Only methods in routine use in two or more UK diagnostic laboratories were tested. This therefore excluded, for example, pulsed field gel electrophoresis. The following methods were compared: microsatellite analysis, detection of the common junction fragment by Southern blotting, detection of the common junction fragment by polymerase chain reaction (PCR), and dosage analysis of *PMP-22* using semi-quantitative fluorescent PCR (STS dosage) with products analysed using either

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Table 1 PCR primer sequences for STS dosage analysis

Primer	Sequence* (5'-3')	Product size (bp)
PMP-22 Pm1A F	GTC TTG GCA TCA CAG GCT TCA GGC A	268
PMP-22 Pm1A R2	ACC AGG CTC CCC GAG ATG TTC CCT G	
PMP-22 Ex4 F	CAT CTC AAC TCG GAT TAC TCC TAC G	374
PMP-22 3'UTR R1	AGG CTC AAC ACG AGG CTG ATG GTC A	
PMP-22 3'UTR F	CTG CTT TTG TAC CTA GCT AGG CTG C	306
PMP-22 3'UTR R3	ATG CAT CTT AGT CCA CAC AGT TGG	
P _o Ex1 F	CAG TGG ACA CAA AGC CCT CTG TGT A	389
P _o Ex1 R2	GAC ACC TGA GTC CCA AGA CTC CCA G	
P _o Ex2 F	CTC ACT TCC TCT GTA TCC CTT ACT G	393
P _o Ex2 R	GGA GGA CAA TGT AGT CAG GGT GAC A	
P _o Ex3 F	TGA CAG CTG TGT TCT CAT TAG GGT C	453
P _o Ex3 R	TCC GAG TGT ATG CCC TGC ATT GAG G	

*Sequences from refs 4, 20–23.

capillary electrophoresis or polyacrylamide gel electrophoresis (PAGE).

SAMPLE DISTRIBUTION

One hundred samples referred to UK Genetics Centres for HMSN testing were collected and comprised approximately 50 samples in which a duplication had been found previously and 50 samples in which a duplication had not been found previously. Patient consent was not sought as the samples had been obtained for the purpose of HMSN testing in the first instance. Samples were classified according to the methods used locally by the contributing laboratories, such as the detection of junction fragments. Consequently, samples in which a duplication had not been detected were not necessarily duplication negative. Several DNA extraction methods had been used by the contributing laboratories, including salting out with ammonium acetate (32 samples) or sodium chloride (10 samples), phenol chloroform extraction (27 samples), and the use of commercial kits, Nucleon (24 samples) (Nucleon Biosciences, UK) and Puregene (seven samples) (Flowgen Instruments, UK). The samples were coded and distributed for testing. Each method was tested independently by two laboratories.

DETECTION OF THE COMMON JUNCTION

FRAGMENT BY SOUTHERN BLOT

This method detects the 3.2 kb *EcoRI/SacI* junction fragment specific to duplications. A total of 5–10 µg of genomic DNA was digested with *EcoRI* and *SacI* according to the manufacturer's instructions, blotted, and probed. The probe PLR6.0, which corresponds to the 6.0 kb *EcoRI* fragment of the distal CMT1A-REP, was used by one laboratory and the probe pNEA102, corresponding to a 1.8 kb end fragment of PLR6.0, was used by the second.^{2 10 11} Both probes detect a 6.0 kb fragment from the distal CMT1A-REP and a 5.0 kb fragment from the proximal CMT1A-REP. PLR6.0 also detects a 2.8 kb fragment from the proximal CMT1A-REP. With both probes HMSN patients have an additional 3.2 kb junction fragment. Samples were classified as duplicated if the junction fragment was detected or duplication not detected if the junction fragment was absent. For samples in which a duplication had not been detected, analysis of the intensity of the 6.0 kb and 5.0 kb bands¹² was not attempted.

DETECTION OF THE COMMON JUNCTION

FRAGMENT BY PCR

A 3.6 kb region including the *EcoRI* site of the distal CMT1A-REP was amplified. Long range PCR was carried out using a forward primer complementary to both the proximal and distal CMT1A-REPs and a reverse primer complementary to the proximal CMT1A-REP only.¹³ Sequences from the proximal and recombinant CMT1A-REPs only are amplified. Following restriction enzyme digestion with *EcoRI*, the recombinant CMT1A-REP, which contains the *EcoRI* site of the distal CMT1A-REP, is cut into products of 3.2 kb and 0.4 kb. The proximal CMT1A-REP, which lacks the *EcoRI* site, remains intact. Samples were classified as duplicated if the junction fragment was detected or duplication not detected if the junction fragment was absent.

MICROSATELLITE ANALYSIS

The microsatellite markers Mfd41 (D17S261), RM11-GT (D17S122), AFM200yb12 (D17S839), AFM317yg1 (D17S955), and AFM191xh12 (D17S921) were used in the study. The order of the microsatellites with respect to PMP-22 is cen - Mfd41 - RM11-GT - PMP-22 - AFM200yb12 - AFM317yg1 - AFM191xh12 - tel.^{14–16} Amplifications were carried out as previously described.^{1 17 18} PCR products were visualised by silver staining following PAGE in one laboratory. Fluorescently labelled PCR products were detected by the second laboratory using an AB 373 sequence analyser (Applied Biosystems, USA). Samples were classified as duplication detected by the presence of three different alleles with a single marker or by a 2:1 dosage ratio between two alleles observed with at least two markers. Samples were classified as duplication not detected if a 1:1 dosage ratio between two alleles was observed with at least two markers. Dosage estimates were subjective in both laboratories. Dosage determination was not always possible if alleles differed by 1 bp or 2 bp owing to interference from stutter bands from the larger allele or if alleles were of substantially differing sizes because of problems of preferential amplification of the smaller sized allele. Such results were scored as non-reportable. A result was scored as uninformative if only one allele was observed.

GENE DOSAGE BY SEMI-QUANTITATIVE PCR (STS DOSAGE)

This method involves direct detection of duplication of PMP-22 using semi-quantitative fluorescent multiplex PCR.¹⁹ Three sequence tagged sites from PMP-22 (Pm1A, exon 4, and 3'UTR) and three exons (exons 1, 2, and 3) from a control gene, myelin protein zero (P_o) on 1q22-23, were coamplified in a fluorescent multiplex PCR. A total of 2–10 pmol of each primer, 100–200 ng of genomic DNA, and 18 PCR cycles were used to ensure the reaction was kept within the exponential phase. The primer sequences are shown in table 1.^{4 20–23}

The products were resolved on either an AB (Applied Biosystems, USA) 373 or 377 sequence analyser (PAGE) or 310 sequence ana-

Table 2 Comparison of the results obtained from the two laboratories testing by the same method

Method	No of analyses compared	No of analyses discordant (%)
Junction fragment by Southern blot	92	2 (2.2)
Junction fragment by PCR	91	0 (0)
Microsatellite analysis	79	2 (2.5)
STS dosage with capillary electrophoresis	89	0 (0)
STS dosage with PAGE	83	4 (4.8)

Table 3 Comparison of the results obtained from the five methods

Method	No of duplications* missed by both laboratories (%)	No of samples† with no result from both laboratories (%)
Junction fragment by Southern blot	7 (14%)	0
Junction fragment by PCR	8 (16%)	1 (1)
Microsatellite analysis	1 (2%)	4 (4.2)
STS dosage with capillary electrophoresis	0 (0%)	0
STS dosage with PAGE	0 (0%)	0

*Total number of samples classified as duplicated = 49.

†Total number of samples = 95.

lyser (capillary electrophoresis) according to the manufacturer's instructions.

The results from the test sample were compared with those from a non-deleted, non-duplicated control sample. Dosage quotients for each STS with each control exon were calculated using peak heights or peak areas, as described previously for dosage analysis of the dystrophin gene.²⁴ An example of the dosage quotient for *PMP-22* exon 4 compared with *P₀* exon 1 is shown.

$$\frac{PMP-22 \text{ exon 4 (test sample)}/P_0 \text{ exon 1 (test sample)}}{PMP-22 \text{ exon 4 (control sample)}/P_0 \text{ exon 1 (control sample)}}$$

A total of nine dosage quotients were calculated for each test sample. The mean and standard deviation of the nine dosage quotients was calculated and a qualitative result, duplicated or not duplicated, determined. If the standard deviation was greater than 10% of the mean the test was repeated.

Results

REPRODUCIBILITY OF METHOD

To test the reproducibility of each method, a comparison was made between the results obtained from the two laboratories testing by the same method. A comparison was therefore only possible if a result was obtained from both laboratories. The number of results compared for each method is shown in table 2. One hundred percent concordance was observed for the detection of junction fragments by PCR and dosage analysis of *PMP-22* with capillary electrophoresis. Discrepancies were observed with the other three methods. The percentage of discordant results observed was 2.2% for the detection of junction fragments by Southern blot (two samples), 2.5% for microsatellite analysis (two samples), and 4.8% for STS dosage analysis with PAGE (four samples). The correct result for the eight discrepant samples was taken as the consensus result from the other four methods. Laboratories with the discrepant results were asked to retest a blinded series of the original samples that included the samples with the discrepant results. Upon retesting, three of the samples failed to give a

result, four samples typed as the consensus result, and one gave the same discrepant result. For the three samples that failed upon retest, it was not possible to determine the cause of the discrepant result. Of the four samples that typed as the consensus result upon retesting, processing errors were given as the cause of the original incorrect result for three samples and one was found to have typed correctly in the first instance but to have been reported incorrectly. One sample retyped with the same discrepant result using dosage analysis with PAGE.

SENSITIVITY OF METHOD

A comparison was made between the results obtained from the different methods in order to test the sensitivity of each method. Identical results were obtained from all 10 laboratories for 44 samples. An additional seven samples were classified as duplication positive by microsatellite analysis and STS dosage methods, but a duplication was not detected by either junction fragment method. For the remaining samples a result was not obtained from one or more laboratories because of either test failure (no result) or the result being uninformative. These samples were classified as duplication positive if evidence for a duplication was seen by two or more methods. Ninety five samples were classified duplication positive or negative. The remaining five samples were withdrawn from the comparison because of indeterminate results (see discussion). To carry out the comparison, a method was scored as having achieved a result if a result was obtained from at least one of the laboratories testing by that method.

Forty nine samples were classified as duplication positive. Dosage analysis of *PMP-22* using semi-quantitative fluorescent PCR with either capillary electrophoresis or PAGE detected all the duplications (table 3) and 48/49 (98%) of duplications were detected by microsatellite analysis. The remaining duplication detected by STS dosage methods was confirmed by methods detecting junction fragments. Microsatellite analysis failed to detect a duplication in this sample as informative results were obtained from only one marker. One laboratory found a 2:1 dosage ratio between alleles with mfd41. The results from AFM191xh12, AFM200yb12, and AFM317yg1 were uninformative and the results from RM11GT were deemed non-reportable. Reporting on a single marker would have given the correct result but fell below the internal quality control criteria set by the laboratory. The second laboratory found a normal dosage ratio with RM11GT. The results from AFM191xh12 and AFM200yb12 were uninformative and analysis with mfd41 failed. Reporting on a single marker in this instance would have led to an incorrect result.

Fourteen percent of duplications were not detected by methods used to detect the common junction fragment. Analysis of one sample failed for both laboratories detecting junction fragments by PCR resulting in 16% of duplications being undetected. Microsatellite

Table 4 Number of failed tests and repeat analyses for each method

Method	No of failed tests*	No of samples analysed more than once
Junction fragment by Southern blot	10	9
Junction fragment by PCR	13	11
Microsatellite analysis	26	27
STS dosage with capillary electrophoresis	12	64
STS dosage with PAGE	20	49

*Total number of failed tests = 81.

analysis and STS dosage methods detected all of the duplications missed by junction fragment methods.

Forty six samples were classified as duplication negative by junction fragment and STS dosage methods. Microsatellite analysis failed to produce a result for three samples because of either uninformative results or inconsistent results between markers.

TEST FAILURE AND REPEAT ANALYSIS

Out of a total of 1000 tests, 81 failed (that is, null or uninformative results, table 4). Both microsatellite analysis and STS dosage with PAGE had more failures than the other methods.

The number of samples analysed more than once is also shown in table 4. The number of repeat analyses does not include repeat analysis on samples that ultimately failed. The STS dosage methods required repeat analysis more often than the other methods.

POLYMORPHISM IN *PMP-22* EXON 4 AMPLICON

For one sample a split peak was observed with the *PMP-22* exon 4 amplicon. The two peaks differed in size by 5 bp. The amplicon was sequenced (data not shown; EG, personal communication) and the difference found to be the result of a polymorphism in the number of repeats of CAAAC present in the 3'UTR of the gene. The sample was found to be heterozygous for two and three copies of the repeat.

Discussion

Five methods routinely used in UK diagnostic laboratories to detect the HMSN1A 17p11.2 duplication have been evaluated on a series of 100 DNA samples. Eight samples had discrepant results between the two laboratories testing by the same method. Where the reason for the discrepancy was determined, it was found to be the result of processing or typographical errors. These errors were therefore the result of problems in carrying out the method and not problems inherent in the method. One sample retyped with the same discrepant result, using STS dosage with PAGE. The reason for this

could be a processing error or a problem inherent in the method. Ninety five samples were classified by two or more methods as either duplication positive (49 samples) or duplication negative (46 samples). The remaining five samples had indeterminate results (table 5). Sample 100 had results for microsatellite analysis only (not duplicated). Sample 33 typed as not duplicated by microsatellite analysis with distal and proximal markers, but typed as duplicated by dosage analysis with capillary electrophoresis. These sample were withdrawn from the comparison of methods as they were not classified by two or more methods.

Three samples may have a duplication smaller than 1.5 Mb.²⁵ Sample 38 typed as duplicated by both STS dosage methods but did not have the common junction fragment by both junction fragment methods. Results from microsatellite analysis were inconsistent; both laboratories found the sample to be duplicated with the proximal marker RM11GT but not duplicated with the distal marker AFM191xh12. This sample could have a duplication that includes RM11GT but does not include AFM191xh12. Sample 1 typed as duplicated by both STS dosage methods but did not have the common junction fragment by both junction fragment methods. Microsatellite analysis found the sample to be not duplicated with the two proximal markers and the most distal marker (AFM191xh12). The closest distal marker to *PMP-22* (AFM200yb12) was uninformative. If this sample has a partial duplication then it must occur between RM11-GT and AFM191xh12. Sample 94 typed as duplicated by dosage analysis with capillary electrophoresis and junction fragment detection by Southern blot but typed as not duplicated by microsatellite analysis. This sample was informative for distal microsatellites only. Results were not obtained from the other methods.

One sample, classified as duplicated, was found to have a polymorphism in the *PMP-22* exon 4 amplicon. This sample was found to be heterozygous for two and three copies of a 5 bp repeat in the 3'UTR of the gene.

A diagnostic test needs to be relatively easy to perform, have a high detection rate, and not be prone to test failure or uninformative results. The use of methods to detect the common junction fragment detected 86% of duplications (table 3). This is consistent with the detection rate reported in previous studies. Screening for the *EcoRI/SacI* junction fragment by Southern blot is reported to detect about

Table 5 Summary of samples with indeterminate results

	Sample No				
	100	33	38	1	94
Junction fragment by Southern blot	F	F	N	N	D
Junction fragment by PCR	F	F	N	N	F
Microsatellite analysis	N	N	I	N	N
STS dosage with capillary electrophoresis	F	D	D	D	D
STS dosage with PAGE	F	F	D	D	F
Classification	Withdrawn	Withdrawn	Possible partial duplication	Possible partial duplication	Possible partial duplication

F = failed. N = not duplicated. D = duplicated. I = inconsistent.

80% of duplications.¹⁰⁻¹² Screening for the same rearrangement by PCR is also reported to detect 80% of duplications.¹³ The results from the PCR method compare with those from the Southern blot method. PCR may therefore be the method of choice for the detection of junction fragments as it is quicker to perform, needs less DNA, and does not require the use of radioactivity.

Overall, the use of microsatellites failed to yield a result in 4.2% of classified samples (table 3). Microsatellite analysis often requires several tests in order to obtain an informative result. The failure to obtain a result was because of the results being non-reportable or uninformative as well as failure to amplify DNA from the sample.

The use of STS dosage was the most sensitive method (table 3). Results were obtained from at least one laboratory for all of the classified samples with both PAGE and capillary electrophoresis. However, this approach had a high failure rate and required more repeat analyses than the other methods, perhaps reflecting a greater technical difficulty (table 4). For both methods, the test was repeated if the standard deviation of the dosage quotients was greater than 10% of the mean. With capillary electrophoresis the sample is resolved through a polymer and the whole sample loading is analysed. With PAGE the sample is resolved through a gel and a reading is taken through a transect of the sample track. The two electrophoresis systems were compared to determine if analysing data from the whole sample is more robust than analysing data through a transect of the sample track where equivalent data points may not be compared owing to gel variation during electrophoresis. This would affect the dosage quotient calculation and increase the standard deviation. A greater number of test failures was observed with PAGE (20 samples) than with capillary electrophoresis (12 samples), which suggests that capillary electrophoresis is more robust than PAGE for the detection of gene dosage abnormalities.

Of the 100 samples tested, 15 failed to give a result in more than one laboratory (data not shown). The samples tested were extracted in several laboratories by a variety of methods (see sample distribution). Fewer failures were observed with DNA extracted using salt purification methods. However, as these samples may also be the most recently extracted, few conclusions can be drawn regarding test failure and extraction method. The failure to obtain a

result is either test dependent, sample dependent, or both. For microsatellite analysis, reasons for test failure were mostly test dependent and included uninformative results, informative results from a single marker only, and inconsistent results between microsatellites. A total of 26 test failures were observed with microsatellite analysis, 15 of which were because results were obtained from a single marker only. Had the result been reported it would have been incorrect in 3/15 cases. RM11GT has been found not to give consistent results in quantitative dosage analyses, and in some laboratories it is only used to detect the presence of three alleles (DEB, personal communication). The addition of further microsatellite markers from within the duplicated region may improve the sensitivity of this approach (and make it more likely that three alleles will be observed in duplicated samples), but would also increase the number of tests required to obtain a result. For STS dosage methods the main reason for test failure was poor data which is both test and sample dependent. In addition, poor data resulted in repeat analysis being carried out for several samples which may reflect the performance of the test with poor quality samples.

The advantages and disadvantages of each method are summarised in table 6. The method of choice for detecting the 17p11.2 duplication will ultimately depend upon the facilities available and the limitations of each test. For the less sensitive methods, the limitations of the test sensitivity should be reported with the results. Patients with hereditary neuropathy with liability to pressure palsies (HNPP) can also be screened with the same molecular genetic techniques to detect the reciprocal 1.5 Mb deletion at 17p11.2-p12. HMSN and HNPP patients that are negative for the duplication/deletion test should be screened for point mutations in the most relevant peripheral myelin protein genes.

In conclusion, this study showed that STS dosage analysis by semi-quantitative PCR was the most sensitive assay for the detection of *PMP-22* duplications, but is technically more demanding than the other methods. Alternative gene dosage methods such as array comparative genomic hybridisation (array-CGH)²⁶ or multiplex amplifiable probe hybridisation (MAPH)²⁷ might retain the high sensitivity that we observed in a simpler assay format.

Table 6 Summary of the advantages and disadvantages of each method

Method	Advantages	Disadvantages
Junction fragment by Southern blot	Low failure and repeat analysis rate	Radioisotope used in assay 14% of duplications missed
Junction fragment by PCR	Rapid Low failure and repeat analysis rate	14% of duplications missed
Microsatellite analysis	Samples can be classified as duplicated or not duplicated	High failure rate Several tests required Partial duplications may be missed
STS dosage with capillary electrophoresis	Samples classified as duplicated or not duplicated	Requires expensive equipment Repeat analysis often necessary
STS dosage with PAGE	Samples classified as duplicated or not duplicated	Requires expensive equipment Repeat analysis often necessary

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