

## ORIGINAL ARTICLE

# Measurement of the clinical utility of a combined mutation detection protocol in carriers of Duchenne and Becker muscular dystrophy

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Supplementary tables 1, 2 and 3 are available online at <http://jmg.bmj.com/supplemental>

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**Background:** Recent methodological advances have improved the detection rate for dystrophin mutations, but there are no published studies that have measured the clinical utility of these protocols for carrier detection compared with conventional carrier testing protocols that use pedigree, serum creatine kinase levels and linkage analysis.

**Methods and subjects:** The clinical utility of a combined mutation detection protocol was measured. It involved quantitative PCR procedures followed by DNA sequence analysis for the identification of dystrophin mutation carriers in 2101 women at risk of being carriers from 348 mutation-known Duchenne or Becker muscular dystrophy pedigrees.

**Results:** The combined mutation detection protocol identified a mutation in 96% and 82% of index cases of Duchenne muscular dystrophy and Becker muscular dystrophy, respectively. An additional 692 (33%) potential carriers were correctly classified by the combined mutation detection protocol compared with pedigree, serum creatine kinase levels and linkage analysis. Significantly lower mutation carrier rates were identified in the mothers of isolated cases with deletion mutations than predicted from theoretical considerations, but these findings were not confirmed for duplication and DNA sequence mutations.

**Conclusions:** There are significant clinical benefits to be gained from a combined mutation detection protocol for carrier detection. It is recommended that mutation-specific carrier frequencies for the different classes of dystrophin mutations should be taken into account in genetic counselling practice.

Duchenne and Becker muscular dystrophies (D/BMD) are allelic disorders of skeletal muscle in which the major clinical feature is progressive muscle weakness (Online Mendelian Inheritance in Man (OMIM) 310200 & 300376).<sup>1</sup> The clinical diagnosis of D/BMD can be confirmed either by the identification of a mutation in the dystrophin gene or by histological analysis of a patient's affected muscle tissue; however, DNA testing has been found to be more acceptable than muscle biopsy owing to its less invasive nature, minimal side effects and reduced costs. The potential benefits of identifying a dystrophin mutation in a male with symptoms include the confirmation of the clinical diagnosis as the basis for management, the ability to determine the carrier status of female relatives and possibly as a future precondition for therapy.

The standard molecular genetic protocol for the diagnosis of D/BMD is multiplex PCR of a subset of dystrophin exons.<sup>2–3</sup> This technique is able to detect ~95% of deletion mutations, but is unable to detect exon duplications and DNA sequence mutations in males with symptoms. Carrier testing in diagnostic laboratories has been principally based on linkage studies, initially using restriction fragment length polymorphisms detected by Southern blot analysis,<sup>4</sup> and subsequently by PCR amplification of short tandem repeat (STR) loci.<sup>5</sup> Linkage

studies can be used to identify deletion mutation carriers on the basis of the presence of an informative STR locus within the deletion interval, and the demonstration of either heterozygosity or apparent non-Mendelian inheritance, but are unable to determine maternal carrier status for duplication or DNA sequence mutations.<sup>6</sup> As linkage results are frequently compromised by the unavailability of DNA samples, the distribution and informativeness of STRs, and the possibility of gonadal mosaicism, adjunct techniques such as fluorescence in situ hybridisation and pulsed field gel electrophoresis have been used by reference laboratories to resolve such cases.<sup>7</sup>

The recent introduction of quantitative PCR-based techniques, such as multiplex ligation-dependent probe amplification (MLPA), have significantly improved mutation detection for exon deletions and duplications in males with symptoms and carrier females.<sup>8–9</sup> Similarly, point mutation detection has been improved by the availability of techniques such as detection of virtually all mutations, denaturing gradient gel electrophoresis,

**Abbreviations:** BMD, Becker muscular dystrophy; CK, creatine kinase; D/BMD, Duchenne and Becker muscular dystrophies; DMD, Duchenne muscular dystrophy; MLPA, multiplex ligation-dependent probe amplification; qPCR, quantitative fluorescent PCR technique; STR, short tandem repeat

denaturing high-performance liquid chromatography and protein truncation test, but the falling costs of DNA sequencing and the availability of semi-automated DNA analysis software have made direct DNA sequence analysis of the dystrophin gene feasible.<sup>10</sup> Mutation detection protocols based on the sequential analysis of samples by MLPA and DNA sequence analysis therefore have the potential for high mutation detection sensitivities. While there are several published studies that address issues of analytical and clinical validity of either MLPA or DNA sequence analysis for dystrophin mutation detection, studies that have measured the clinical utility of combined MLPA and DNA sequence analysis compared to that of conventional protocols for carrier detection available to non-reference laboratories are lacking.

## SUBJECTS AND METHODS

Subjects were identified in a review of D/BMD pedigrees ascertained over the past 35 years through neuromuscular disorders clinics in New South Wales, Australia. All dystrophin analyses were performed at a single reference laboratory between 1 March 1985 and 31 December 2006. This review was conducted with the approval of the South Eastern Sydney Illawarra Area Health Service Research Ethics Secretariat.

Three cohorts are reported in this publication. The first cohort includes 481 index cases used to determine the analytical performance of a mutation detection protocol, which includes an exon deletion screen using either the Chamberlain and Beggs multiplex PCR sets, an in-house quantitative fluorescent PCR technique (qfPCR) based on the method of Yau *et al*<sup>9</sup> or MLPA (MRC-Holland, Amsterdam, The Netherlands, cat# P034/P035), followed by DNA sequence analysis of all coding exons and splice junctions of dystrophin using an ABI3730xl Genetic Analyser (Applied Biosystems, Foster City, California, USA).

The second population is a subgroup of 348 families of the above cohort that was used to determine the clinical utility of carrier testing. Subjects were the female relatives of individuals affected with D/BMD whose mutation had been identified by the above protocol, and for whom linkage data were available. To estimate the additional clinical benefit of combined mutation detection in carriers, we reviewed all information available to the laboratory that related to the carrier status of these women including pedigrees, clinician letters, serum creatine kinase (CK) levels, linkage results, the results of quantitative analyses (either an in-house multiplex qfPCR assay or a commercially available MLPA kit) and the results of DNA sequence analysis. For each female family member, the earliest point in the hierarchy of investigations at which her carrier status was correctly assigned was determined.

The third population comprised 201 mothers of patients with Duchenne muscular dystrophy (DMD) referred for initial diagnosis at this laboratory, and whose samples were investigated to determine whether mutation type had an effect on carrier rates. Cases were required to be mutation-known and isolated as determined from a complete three-generation pedigree. The carrier status was defined by genetic testing results, or inferred from markedly elevated CK levels.

Statistical analyses were performed using the statistical software package SPSS v14.0.

## RESULTS

### Mutation detection

Mutation detection was performed on a retrospective cohort of 481 index cases, of which 394 were classified as DMD and 87 as Becker muscular dystrophy (BMD). DNA was available from a male with symptoms in 387 (80%) cases and from 94 (20%) female family members. Table 1 gives a summary of the

number of cases for each mutational class by diagnosis and analytical technique (the complete dataset is available online at <http://jmg.bmj.com/supplemental>). Overall, the mutation detection rate was 96% for DMD pedigrees and 82% for BMD pedigrees. The use of a combined qfPCR/MLPA and DNA-sequencing protocol in this cohort identified an additional 248 mutations over those identified by the Chamberlain and Beggs multiplex PCR sets, of which 144 were initially detected by qfPCR/MLPA and 104 by DNA sequencing.

$\chi^2$  analysis of the mutation results indicate that there are significant differences between the mutation frequencies in this cohort compared with those reported in similar mutation surveys<sup>11–13</sup> ( $\chi^2_{df\ 6} = 20.7$ ,  $p \leq 0.01$ ), or in the Leiden locus-specific database ( $\chi^2_{df\ 2} = 57.7$ ,  $p \leq 0.001$ , date of query 3 January 2007). These results are predominantly driven by the high frequency of duplication mutations in the New South Wales population, which at 14% is 3–9% higher than that reported for other clinical populations. A report of a high duplication rate from a Chinese population suggested that this result might be due to population admixture.<sup>14</sup> Review of our data identified four duplication mutations in 12 north Asian index cases (33%, 95% CI 6 to 50), which is insufficient to account for the observed excess of duplication mutations.

### Carrier testing

Determination of the carrier status in female relatives is a significant component of providing service in diagnostic laboratories. We undertook to establish the clinical utility of the combined qfPCR/MLPA and DNA-sequencing protocol for carrier testing by directly measuring the number of additional carriers that could be identified compared with the established protocol of pedigree, CK and linkage analyses. A total of 348 pedigrees were reviewed and the carrier status of 2101 female relatives of 616 affected individuals was determined on the basis of pedigree analysis or test results (table 2, and supplemental table 2 available online at <http://jmg.bmj.com/supplemental>).

The carrier status of 1409 women (67% of the tested group) could be assigned on the basis of the pedigree analysis, the presence of a raised CK and/or linkage studies that showed either non-Mendelian inheritance, heterozygosity of alleles within the deletion region or inheritance of a normal allele.

The carrier status could be determined by pedigree analysis alone in 224 (11%) women including 9 manifesting carriers, 198 obligate carriers by pedigree and 17 women with  $\geq 3$  affected progeny. An elevated CK classified 233 women as carriers by comparison with laboratory-specific reference ranges. Women were included in this group only when it could be established from clinical records that they had been informed that they were carriers based on CK results. Significantly, 32 assignments by CK were subsequently shown to be false positives after mutation testing, of which 50% were CK levels in the range 250–1200 U. Taking the false positives into account, CK levels accurately classified an additional 201 (10%) carriers.

The carrier status of a further 984 (47%) women could be assigned by linkage analysis, with over half of these being excluded as carriers based on the linkage results of another female relative without recourse to testing. Linkage analysis was most productive among first- and second-degree relatives in families where there were several affected individuals, and least effective in families of isolated cases due to non-deletional mutations.

The combined mutation detection protocol determined the carrier status of a further 692 women who were uninformative by standard techniques (314 by testing, and 378 by exclusion following a negative result of another relative). The use of

**Table 1** Summary of mutational classes observed by diagnosis and analytical method

Diagnosis and mutational class	Method of mutation deletion								Total (%)
	Exon mPCR		qfPCR/MLPA		DNA sequence		Mut <sup>n</sup> negative		
Duchenne	M	F	M	F	M	F	M	F	
Exon deletions	162		29	37					228 (58)
Exon duplications			31	24					55 (14)
DNA sequence mutations	1		3		71	21			96 (24)
Mutation negative							10	5	15 (4)
DMD mutations									394
	379 (96%)								
Becker									
Exon deletions	39		6	6					51 (59)
Exon duplications			8						8 (9)
DNA sequence mutations					11	1			12 (14)
Mutation negative							16		16 (18)
BMD mutations									87
	71 (82%)								

BMD, Becker muscular dystrophy; DMD, Duchenne muscular dystrophy; F, female; M, male; MLPA, multiplex ligation-dependent probe amplification; mPCR, multiplex PCR; qfPCR, quantitative fluorescent PCR.

qfPCR/MLPA classified 534 (25%) women either by direct testing or as the result of exclusion. DNA sequence analysis determined the carrier status of 158 (8%) women. The additional benefit of combined mutation detection was greatest among first-degree relatives, whereas the benefit of exclusion power was greater for more distant relatives.

Review of the pedigrees indicated that there were 967 additional named females at risk, principally from early generations, where testing had not been performed owing to the lack of a DNA sample, but whose status could have been resolved by these protocols. There were also 28 prenatal diagnoses performed by linkage for women whose carrier status was uncertain, but were subsequently shown not to be carriers. If it is assumed that a quarter of such fetuses would be affected male embryos, approximately six normal male pregnancies would have been at risk of termination. Laboratory records do not indicate actual outcomes in these cases.

### Maternal carrier rates

The accepted mutation carrier frequency for mothers of isolated cases of DMD has been debated on the basis of several studies where a lower than predicted carrier frequency for deletion mutations was identified in mothers of isolated cases.<sup>15–17</sup> To investigate this issue, the carrier frequency in 201 mothers of isolated cases of DMD was studied. The likelihood that a mother of an isolated case is a carrier of a deletion mutation was found to be 40%, which is significantly lower than the

predicted frequency of two-thirds<sup>18</sup> for a X linked lethal disorder ( $p < 10^{-8}$ ) and consistent with the literature cited above. No difference in the involvement of the major compared to minor deletion clusters was identified ( $\chi^2_{df=1} = 0.55$ ,  $p = \text{NS}$ ).<sup>19</sup> This result was in clear contrast with the carrier frequency for the other classes of mutations studied, which were found to be not significantly different from the predicted value (table 3, and supplemental table 3 available online at <http://jmg.bmj.com/supplemental>).

### DISCUSSION

In this study, we have measured the clinical utility of a combined mutation detection protocol for D/BMD in a large cohort of families analysed at a single referral centre using one or more quantitative PCR-based procedures followed by DNA sequence analysis. Clinical utility in the broad sense, encompasses a number of related concepts that have an impact on clinical outcome.<sup>20</sup> The measures of clinical utility used here are those, in the narrow sense, associated with clinical end points such as the sensitivity of the protocol for the detection of mutations in affected individuals, the ability to correctly classify female relatives as carriers and the additional numbers of carriers correctly classified by this protocol compared with conventional testing.

Publications concerning the use of combinations of technologies to detect the full mutational spectrum in D/BMD are relatively few in number with only four available for

**Table 2** Categorisation of carrier/non-carrier status by method

Relationship	Result number		Pedigree	↑ CK	Linkage	qfPCR/MLPA	DNA sequencing
1st degree	803	Carrier	181	153	29	63	18
		Non-carrier		10*	221	103	31
		Excluded			4		
2nd degree	604	Carrier	29	38	16	11	5
		Non-carrier		12*	121	39	20
		Excluded			191	110	24
3rd degree	490	Carrier	10	8	2	1	
		Non-carrier		9*	37	7	13
		Excluded			251	137	24
≥4th degree	204	Carrier	4	2			
		Non-carrier		1*	16	2	1
		Excluded			96	61	22
			224 (11%)	201 (10%)	984 (47%)	534 (25%)	158 (8%)
Total	2101			1409 (67%)		692 (33%)	

CK, creatine kinase; ↑ CK, increase in CK; MLPA, multiplex ligation-dependent probe amplification; qfPCR, quantitative fluorescent PCR.

\*False positive CK results are not included in the total of this column.

**Table 3** Mothers of isolated cases: carrier frequency by mutation class

Mutation class	Mothers of isolated cases				p Value
	n	Expected carriers*	Observed carriers	95% CI	
Deletions	140	93.3	56	45.1 to 66.9	$p \leq 10^{-8}$
Duplications	27	17.8	17	12.2 to 21.8	$p = 0.61$
Small dels and subs	34	22.6	19	13.6 to 24.4	$p = 0.17$
Totals	201	133.7	92		

Dels, deletions; subs, substitutions.

\*Assumes a carrier rate of two-thirds.<sup>18</sup>

comparison.<sup>6 12 21 22</sup> Overall, the sensitivities achieved for mutation detection in index cases of our cohort were 96% for DMD and 82% for BMD, which are similar to those reported previously. This corresponds to an additional 248 families for whom mutation information could be provided compared with conventional testing procedures and represents important information that could be significant in determining access to therapeutic interventions in the future. The spectrum of mutations identified differs slightly from those reported in similar studies and in the D/BMD mutation databases of the Leiden Muscular Dystrophy Pages<sup>23</sup> with a significantly higher duplication frequency in the New South Wales population (14%) compared with similar studies (5–10%). Several authors have reported differences in the frequencies of specific dystrophin mutation classes in different ethnic groups and it has been suggested that this may be due to geographical variation in intronic sequences in the dystrophin gene.<sup>14 24–27</sup> The possibility that duplications are over-represented in one ethnic group could not be rigorously investigated owing to the lack of information regarding the ancestry of index cases in the majority of our cohort. The frequency of other classes of mutations were not significantly different from those that were previously reported.

For DMD, a major component of providing genetic service relates to carrier detection in the female relatives of an affected male. This has implications for the uptake of prenatal diagnosis in subsequent pregnancies, and whether screening for dilated cardiomyopathy should be undertaken. The main objective of this study was to measure any improvement in carrier detection rates obtained through the use of the combined mutation detection protocol, compared with that available from conventional procedures based on pedigree analysis, measurement of CK and linkage analysis. We have shown that combined mutation detection protocols have significant advantages over conventional methods for the determination of mutation carrier state, with at least an additional 33% of female relatives of an affected individual having their carrier status clarified.

The cost of the initial mutation detection in the index case is high, but this is offset by the accuracy of mutation carrier detection, which enables other costs to be avoided. These include the costs of cardiac surveillance, prenatal diagnoses and diagnostic muscle biopsies. In this cohort, there were 424 first and second-degree female relatives who were uninformative by conventional testing, of which 327 (77%) were shown not to carry a mutation as a result of this protocol and could therefore be excused further cardiac assessment, prenatal diagnoses and follow-up. In addition, there are a percentage of index cases of D/BMD who, in the absence of a positive deletion screen result, could only be diagnosed by testing affected muscle obtained at open biopsy. The true costs of day surgery and histological examination of tissue and/or western blot analysis are substantial, and in real terms may exceed that of mutation screening using the combined protocol. The number of patients for whom muscle biopsy is now relevant has been markedly reduced through this approach.

Current genetic counselling practice is to cite a maternal carrier risk of two-thirds for the mother of an isolated case of DMD.<sup>18</sup> This risk for an X linked disorder with early lethality assumes that there is equilibrium between mutation and selection, the mutation rates for all mutational classes observed to cause DMD are the same in the ova and the sperm, and carrier women have the same reproductive fitness as non-carrier women. Four studies have now reported reduced carrier rates in mothers of isolated cases of dystrophin deletion mutations.<sup>15–17 28</sup> Although the numbers for each study are individually small (23–41 cases), the combined data indicate a carrier frequency of 27% (33/122), significantly lower than the predicted 67%. In this study, we confirm that the carrier rate for the mothers of isolated affected males with deletions is substantially lower than predicted at 40% of potential carriers in this cohort. Surprisingly, we found that the decreased carrier rates are restricted just to deletion mutations, with the likelihood of being a carrier for the other classes of mutations being in the range 55–63%. These findings indicate that the assumptions underlying the carrier risk calculations for the mother of an isolated case of DMD are not valid for deletion mutations, but do appear to be valid for other mutational classes and suggest a basic biological difference in the effect of a deletional mutation compared with a non-deletional mutation. Possible reasons for this departure from the theoretical risk are that the mutation rates in the ova are not the same for all classes of dystrophin mutations, with higher mutation rates having been suggested for deletions compared with DNA sequence and duplication mutations.<sup>29</sup> An alternative hypothesis is that there is a difference in the viability of gametes or early embryos with deletion mutations.

In summary, this project has shown that a combined mutation detection protocol that includes a quantitative PCR procedure such as MLPA followed by DNA sequencing of dystrophin offers measurable advantages over the standard approach of pedigree analysis, CK and linkage studies to determine the carrier status.

## ELECTRONIC DATABASE INFORMATION

Leiden Muscular Dystrophy Pages, <http://www.dmd.nl/>; Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/>

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