The clinical and molecular genetic approach to Duchenne and Becker muscular dystrophy: an updated protocol

Anthonie J van Essen, Alexander L J Knepers, Annemieke H van der Hout, Hans Scheffer, Jeke B Ginjaar, Leo P ten Kate, Gert-Jan B van Ommen, Charles H C M Buys, Egbert Bakker

Abstract
Detection of large rearrangements in the dystrophin gene in Duchenne and Becker muscular dystrophy is possible in about 65-70% of patients by Southern blotting or multiplex PCR. Subsequently, carrier detection is possible by assessing the intensity of relevant bands, but preferably by a non-quantitative test method. Detection of microdeletions in Duchenne and Becker muscular dystrophy is currently under way. Single strand conformational analysis, heteroduplex analysis, and the protein truncation test are mostly used for this purpose. In this paper we review the available methods for detection of large and small mutations in patients and in carriers and propose a systematic approach for genetic analysis and genetic counselling of DMD and BMD families, including prenatal and preimplantation diagnosis.

Keywords: Duchenne muscular dystrophy; laboratory diagnosis; carrier state; mutation

Duchenne muscular dystrophy (DMD) is the most frequent muscle disease in children. The birth prevalence of DMD is around 1 in 3500 live born males. The milder Becker muscular dystrophy (BMD) has a lower birth prevalence of around 1 in 18 500 live born males. Duchenne and BMD are allelic X linked recessive diseases caused by mutations in the dystrophin gene in the middle of the short arm of the X chromosome (Xp21).

Female relatives of DMD and BMD patients, including mothers, sisters, nieces, aunts, and cousins, often request genetic counselling and genetic testing as these diseases are incurable, severely disabling, and cause early death. In the genetic analysis of the family, the first question to be answered is how reliable the diagnosis is in the index case. The certainty of diagnosis in DMD or BMD can be established by the criteria set by Jennekens et al. Subsequently, mutation detection enables carrier detection and prenatal diagnosis in the family. Several molecular methods for detecting deletion heterozygotes are available. However, carrier detection may not be straightforward, as the normal X chromosome usually masks a deletion on the other X. If the mutation cannot be detected, determination of serum creatine kinase (CK) activities in close female relatives and linkage analysis with intragenic and flanking DNA markers can help to determine carrier risks and accomplish prenatal diagnosis.

A World Wide Web site has been set up by Drs J T den Dunnen and E Bakker with scientific and diagnostic data on DMD and BMD, including all detected rearrangements and microdeletions in the dystrophin gene (http://ruly70.MedFac.LeidenUniv.nl/~duchenne/).

In this paper, we propose a systematic approach for genetic analysis in DMD and BMD families requesting genetic counselling and prenatal or preimplantation diagnosis.

Genetics
THE DYSTROPHIN GENE
Spanning 2.4 Mb in Xp21, the dystrophin gene is the largest human gene known to date. It contains 79 exons and codes for a 14 kb mRNA transcript. The protein product, dystrophin, has a molecular weight of 427 kDa. Normally, dystrophin is located underneath the muscle cell membrane, but in DMD patients it is virtually missing (<3%). BMD patients mostly have 10-40% of normal dystrophin or make a smaller or larger, less functional dystrophin protein. In both DMD and BMD, partial deletions and duplications cluster in two recombination hot spots, one proximal at the 5' end of the gene, comprising exons 2-20 (30%), and one more distal, comprising exons 44-53 (70%). The site and size of these mutations are very heterogeneous. DNA analysis enables detection of partial deletions in about 65% of patients with DMD/BMD and partial duplications in 5% of patients. Frameshifting mutations generally cause DMD, while in BMD the reading frame of the gene is usually intact. This frameshift model complies with the phenotype in 92% of cases. Detection of microdeletions like point mutations is under way but not routine yet in most laboratories. Microdeletions are more difficult to find in the incomparably large dystrophin gene as they do not cluster in certain regions of the gene.

MOSAICISM
Germline mosaicism in DMD was first described by Bakker et al. Many reports have followed since. Subsequent brothers and sisters of a patient with an apparent new mutation have an estimated risk of 5-10% of inheriting the same mutation owing to germline mosaicism. Passos-Bueno et al. found different
frequencies of mosaicism for proximal and distal deletions and duplications in the dystrophin gene in familial and isolated cases, indicating a difference in the aetiology of the mutations. They observed a much higher frequency of proximal gene rearrangements in “proven” germline mosaics. No such differences were found in other studies using smaller data sets.\textsuperscript{26, 27} Grandpaternal germline mosaicism was found indirectly by haplotype analysis\textsuperscript{28} and by direct mutational analysis.\textsuperscript{18} Grandpaternal somatic mosaicism was reported by Lebo et al.\textsuperscript{22} Germinal mosaicism for a DMD point mutation was first described by Wilton et al.\textsuperscript{21} Kneppers et al.\textsuperscript{22} assumed that the recurrence risks for apparently new microlesions were in the same range as for larger rearrangements.

Bakker et al.\textsuperscript{20} found evidence of somatic mosaicism in a female carrier passing the at risk haplotype to a daughter who was not a carrier. No evidence for chimerism or triple X, as was postulated by Witkowski,\textsuperscript{11} was found in this case. Somatic mosaicism in the mother of a son with BMD and a carrier daughter was reported by Voit et al.\textsuperscript{24} Bunyan et al.\textsuperscript{25} reported somatic mosaicism in a mother of a DMD patient using FISH. RT-PCR and a polymorphism from the area of the deletion were used to show somatic mosaicism in another mother of a DMD patient.\textsuperscript{19} Many somatic mosaics in whom the cell lines with the mutation include a large proportion of the lymphocytes are likely to escape detection since the mother is usually regarded as a carrier. This should skew the sex ratio of new mutations towards a higher maternal origin.\textsuperscript{24}

Somatic mosaicism in a DMD patient was reported by Uchino et al.\textsuperscript{79} using PCR fibre analysis and by Saito et al.\textsuperscript{26} with immunocytochemistry and PCR of different tissues.

**FAMILY SITUATIONS**

Occurrence of DMD or BMD patients and carriers in a family can have different origins. The various situations are considered here.

A woman with one affected son and no family history of muscular dystrophy is either: (1) a full carrier; (2) a somatic mosaic coinciding with a partially (germline mosaicism) or totally affected germline; (3) a germline mosaic; (4) not a carrier, the ovum from which her son arose contained a new mutation; (5) not a carrier, her diseased son is a somatic mosaic. Depending on the proportion of muscle cells that contain the mutation, a wide clinical spectrum can be expected.

Sisters of a boy apparently affected by a new mutation are at risk of being a carrier because the mother can be a germline mosaic. Subsequent brothers have the same risk of being affected by the same mutation. This risk lies in the order of 5-10%.\textsuperscript{20, 24} We wish to stress that the same risk applies to the sisters of a woman who is apparently a carrier owing to a new mutation.

A woman with more than one affected son and no family history of muscular dystrophy can be: (1) a full carrier; (2) a somatic mosaic including her germline; (3) a germline mosaic.

If a woman has an affected son and also has affected relatives in the maternal line, she is regarded as a definite carrier. A woman with one or more affected brothers but no affected offspring is a possible carrier.

A woman can be a carrier because: (1) she inherited the mutation from her mother who is a carrier; (2) her mother or her father is a somatic mosaic including the germline; (3) her mother or her father is a germline mosaic; (4) the ovum or sperm from which she arose contained a new mutation; (5) she is a somatic mosaic including her germline.

**Methods of mutation detection by DNA analysis**

**DETECTION OF LARGE REARRANGEMENTS OF THE DYSTROPHIN GENE**

**Polymerase chain reaction (PCR)**

For PCR, only 100 ng DNA is necessary and results can be obtained within one day. Selected synthetic oligonucleotide primers amplify specific sequences over a million-fold in vitro. The two commonly used multiplex PCR kits cover 18 exons in the deletion hot spots of the dystrophin gene. They allow detection of 98% of all deletions.\textsuperscript{39-41}

In patients. Deleted exons cannot be amplified and are recognised as absent bands (fig 1). Duplications might be detected under strictly controlled quantitative conditions.

In carriers. Demonstration of heterozygosity or hemizygosity of polymorphic markers within
regions corresponding to the deletion in the patient allows accurate deduction of carrier status (fig 2) and is probably first choice for carrier detection. Microsatellite markers are very useful for this purpose. However, the available markers are not informative in every family. In uninformative families dosage analysis of PCR products in female relatives can be carried out using ethidium bromide staining, or radioactive probes, or automated fluorescent analysis. Stringent test conditions are needed for reliable results. Competitive PCR has recently been described as a method to detect deletion heterozygotes. It involves a comparison of the amplification efficiency of the deleted area in carriers and controls and has been shown to be successful in 80% of families with deletion patients.

Southern blotting
Southern blotting is more labour intensive than PCR, usually takes several days, and requires a minimum of 5 µg genomic DNA. Southern blot analysis using dystrophin cDNA probes has been described by Darras and Francke, Bakker et al, and Mao and Cremer.

In patients. Deletions are recognised as absent bands or band shifts (junction bands or J bands) on the autoradiogram (figs 3 and 4). J bands are found in less than 5% of patients. Duplications appear as a double band intensity in comparison to the normal situation or as band shifts. Genomic probes (cosmids) can also be used to detect J bands, which are found in 81% of precisely mapped deletions. The technically more demanding pulsed field gel electrophoresis (PFGE) or field inversion electrophoresis (FIGE) are methods to separate long DNA fragments produced by rare cutting enzymes and usually show J bands in deletions larger than 20 kb. Alternatively, a newly developed Southern blotting based method uses rare cutting restriction enzymes and electrophoresis of single stranded DNA and detects J bands in almost 80% of patients with deletions and duplications.

In carriers. Deletions or duplications which produce a J band make carrier detection easy and reliable (fig 3). As J bands are found in less than 5% of the patients, carrier detection by Southern blotting is mostly based on band intensities. This requires quantitative blotting conditions (fig 4). Depending on the practical circumstances in the laboratory and the routine of the personnel with quantitative blotting, this method of carrier detection by dosage analysis may have an increased chance of misdiagnosis, especially when the patient cannot be tested.

Reverse transcription PCR (RT-PCR)
Reverse transcription PCR (RT-PCR) transforms mRNA to cDNA, which is then amplified. A fresh blood sample is needed for isolation and amplification of total RNA which contains very low quantities of lymphocyte dystrophin mRNA, resulting from illegitimate

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**Figure 3** The cDNA blot hybridised with probe 63-3,4 shows a deletion of exon 53 (del) in the DMD patient (S) and results in a junction fragment (J). Half intensity of the relevant band in the mother (2) indicates she is a carrier, which is confirmed by the presence of the J band.

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**Figure 4** (A) Pedigree of the family. (B) The HindIII and BglIII cDNA blot hybridised with probe 7b8 show a deletion of exons 45-47 in the BMD patient (2). The mother (1), who is a carrier, shows half intensity of the relevant bands. The sister (3) is not a carrier as she shows identical density for all bands compared with the normal control (C). (C) Graphical representation of the in frame deletion in the patient.
transcription (about one molecule per 500 cells).56

In patients. Deletions and duplications show as abnormally sized products.57

In carriers. Heterozygotes are accurately detected as they show two different sized products.

Fluorescent in situ hybridisation (FISH)
In patients. Fluorescent cosmid clones allow microscopic detection of gross deletions as no signal is detected. A control probe is needed to avoid a conclusion based on false positive results.44 58 59

In carriers. Carriers show one signal per nucleus with cosmid probes from the region corresponding with the deletion plus two control signals.

Immunological analysis of dystrophin in a muscle biopsy
In patients. DMD patients usually lack detectable dystrophin on immunohistochemistry and immunoblotting (<3%). BMD patients have quantitative/qualitative dystrophin aberrations.

In carriers. Carriers may show mosaic expression of dystrophin negative fibres.56–59 However, asymptomatic carriers are less likely to give informative results. The study of differentiating dystrophin positive and negative clonal myoblasts in vitro may be another approach,60 61 which has not gained popularity yet. While it is hardly feasible as a routine examination, it may be a last ditch method for cases where no mutation is found and haplotyping is not informative. Forced myodifferentiation in cultured fibroblasts is a recently developed technique that may also help to find carriers by dystrophin analysis or transcript analysis in transformed clonal fibroblasts.62

From the above mentioned methods only PCR, Southern blotting, and haplotyping are routinely used in most diagnostic laboratories nowadays.

METHODS FOR DETECTING MICRO LESIONS IN THE DYSTROPHIN GENE IN PATIENTS
Several methods are currently being used for scanning for microlesions in genes,63 including the dystrophin gene.15 16 These methods are described below. After a band shift has been noted in the DNA of the patient, purified samples of aberrant PCR bands must be sequenced for the identification of the mutation, as the aberrant band might be caused by either a polymorphism or a pathogenic mutation. Nonsense mutations and frameshifts leading to a stop codon, as well as splice site mutations, can generally be considered pathogenic. For missense mutations the interpretation is more difficult. Criteria for pathogenicity are absence of the mutation in controls, a change of polarity or size of the amino acid in the encoded protein, occurrence of the amino acid change in a conserved domain, or detrimental effect of the mutation in a functional in vitro system or in an animal model.

Figure 5 SSCP analysis shows a band shift in the DMD patient (2). Sequencing showed a stop mutation at position 1697 in exon 12. The mother (1) carries the same mutation, while the grandmother (4) is not a carrier.

Single strand conformation polymorphism (SSCP)
SSCP has the potential to detect all small mutations. A mutation in the single DNA strand causes altered folding and thus altered migration during electrophoresis. In practice, 35–100% of known mutations can be detected as an aberrant band pattern by SSCP and its modifications.64 65 Application of various different electrophoretic conditions increases the chance of finding an aberrant band caused by the mutation (fig 5). SSCP of exons of the deletion prone regions leads to the detection of pathogenic mutations in about 10% of cases.66 67 68 SSCP is rarely used for detection of large rearrangements.72

Heteroduplex analysis (HDA)
In HDA, after denaturation of the amplified fragments, rehybridisation of the single stranded patient DNA and control DNA produces four hybridisation products, one hybrid with two normal strands, one with two abnormal strands, and two with a normal and a mutated strand. These products migrate differently during electrophoresis. Applications to dystrophin gene mutation screening are described by Prior et al71 and Barbieri et al.44

Protein truncation test (PTT)
The PTT screens DMD lymphocyte mRNA for translation terminating mutations by RT-PCR and subsequent in vitro translation/transcription.16 75–78 Truncated dystrophin products are seen in patients with stop mutations (fig 6). Owing to its selectivity for truncating
mutations, the PTT is not useful for mutation detection in most BMD patients in whom stop mutations are rare.

Chemical mismatch cleavage (CMC)
CMC detects single base mismatches containing cytosine or thymidine in heteroduplexes from patient DNA and control DNA. The mismatched bases are chemically modified and thereby create a cleavage site. Applications to dystrophin mutation screening have been described by Roberts et al. and Kilimann et al.80

Denaturing gradient gel electrophoresis (DGGE)
DGGE separates DNA fragments with a CG clamp by their different melting behaviour in a denaturing gradient. DGGE has a very high mutation detection rate81 82 and has recently been used for mutation analysis of muscle and brain specific promoter regions of the dystrophin gene.83

Microlesion detection in carriers
Once the mutation has been found in the patient, reliable carrier detection is possible by sequencing only a short section of the dystrophin gene. Alternatively, when the mutation produces or abolishes a restriction site, this also enables carrier detection for that mutation. In the absence of patient material, close female relatives may be tested directly for aberrant bands. The PTT can also be used for DMD carrier detection when no patient material is available.

Procedure for genetic analysis
Depending on the availability of the patient and the presence of a detectable mutation in DNA of the patient the following approaches are suggested.

Patient DNA available, mutation detectable
by PCR/Southern Blotting
When a deletion or duplication is detected in the patient, quantitative Southern blotting allows for dosage analysis in female relatives with at least two digests with different restriction enzymes (for example, BgIII, HindIII, PvuII) to confirm the presence and extent of the deletion or duplication and to detect carriers. When the deletion or duplication produces a J Band, carrier detection is straightforward. However, most deletion carriers are detected by a decreased intensity of the band corresponding to the deletion in the affected male relatives. In patients, duplications increase band intensity by a 2:1 ratio. Duplication carriers, however, are more difficult to detect as band intensity is only increased by a 3:2 ratio. Interpretation of quantitative blots requires independent examination by at least two qualified persons.

Carrier detection by differences in band intensities should preferably be confirmed using informative polymorphisms in the region corresponding to the deletion. This means that DNA of the parents of the carrier should also be available. Short tandem repeat (STR) markers are very useful for this purpose. Alternatively, another qualitative carrier test can be used, such as PFGE, RT-PCR, or FISH.

Patient DNA available, mutation not detectable with PCR or Southern Blotting
When no dystrophin gene mutation can be detected, immunoblotting in a muscle biopsy from the patient is important to confirm DMD. Immunohistochemistry may differentiate between DMD and BMD. Testing for deficiency of proteins from the transmembrane sarcoglycan complex is indicated in dystrophin positive myopathies84 85 to identify autosomal recessive limb-girdle muscular dystrophy. Mutations in the genes for the sarcoglycan proteins occur in about 10% of patients with DMD-like and limb-girdle dystrophy with normal dystrophin.

In DMD or BMD diagnosed with certainty, SSCP, HDA, PTT, or another test for mutation scanning should be tried to screen the dystrophin gene for a mutation whenever possible. When an aberrant band is found, that band must be amplified and sequenced for a pathogenic mutation. Carrier detection will then also be possible by sequencing this region. When the mutation produces or abolishes a restriction site, restriction analysis can be used as a ready confirmation of carrier status.

In families where the mutation cannot be detected, carrier risks have to be based on the pedigree, serum CK activities in close female relatives, and linkage data using flanking and intragenic markers. In these cases automated calculation programs can be helpful. However, possible recombinations remain a drawback in linkage analysis. The very high intragenic recombination of 12% across the dystrophin gene86 demands combined use of flanking and intragenic markers. Highly informative polymorphisms are the first choice. In the case of inconsistent results in linkage analysis, sampling errors and non-paternity should be borne in mind. About 2/3 of DMD carriers have raised serum CK activities.87 These are also found in about 1/2 of BMD carriers.88 It should be taken into account that raised serum CK activities occur in about 1 in 20 females in the population and that during pregnancy serum CK activities tend to decline.89 90

Patient DNA not available
In families where patient DNA is not available, a search for stored muscle biopsy, Gunther spots,91 or deciduous teeth92 may be worthwhile. When none of these sources of DNA is available, band intensity can be determined in quantitative PCR blots or Southern blots or both in female relatives. Detection of J bands in Southern blots would, of course, be most helpful. If unsuccessful, PFGE, RT-PCR, SSCP, FISH, and PTT can be considered.

Risk calculation
Risk calculation is needed in families where the mutation cannot be detected. Determination of carrier risks may be difficult, particularly in isolated cases. By including serum CK activities in female relatives as well as pedigree and
haplotype information in a Bayesian calculation, carrier risks may be estimated. As already mentioned, the huge dystrophin gene has an estimated recombination across the gene sequence of 12%, 98 99

Risk calculations usually assume an equal mutation rate in spermatoctyes and ooycts. 96 However, Grimm et al 97 suggest that most point mutations arise during spermatogenesis. If so, this would raise the a priori carrier risk of the mother from 67% to at least 76%. Another complicating factor in risk calculations is the presence of germline or somatic mosaicism. Approaches to risk calculation have been described in a few reports, to which the reader is referred. 98 99

Genetic counselling
Ideally, both partners should be counselled before laboratory tests are considered. All available options in their situation should be discussed with them, including prenatal diagnosis and preimplantation diagnosis, with their limitations and potential complications, such as risk of abortion and recombination between markers in the child causing uninformative test results.

Prenatal diagnosis
Chorionic villus biopsy is preferred by most couples opting for prenatal diagnosis. Usually PCR will be used in prenatal diagnosis of male fetuses. PCR results are generally not influenced by low levels (<5%) of maternal contamination. However, in the case of a male fetus in which no mutation is detected, informative polymorphisms in the mother should be used to exclude maternal contamination.

Fetal muscle biopsy in male fetuses at risk may be considered in a situation where linkage analysis is uninformative or recombination has occurred. 100 101 but has an approximately 5% risk of fetal loss. As an alternative, immunological analysis of dystrophin expression after induction of myogenesis in fetal fibroblasts, amniocytes, or chorionic villus cells can be considered. 102 Additionally, RT-PCR of dystrophin mRNA isolated from these transformed cells might help to identify the mutation. 102

Clinical application of non-invasive prenatal DNA diagnosis of fetal cells in maternal blood is being studied. 103 This type of approach, however, still faces major general problems. 104 105

Prenatal diagnosis (PDD)
In PID one or two cells are removed from the 6-8 cell pre-embryos three days after conception. In a nested PCR, the target exons of these cells are amplified for sex determination or mutation detection. Important drawbacks of PID are that in 20% of cases single cell PCR fails to produce sufficient DNA for analysis and that the chance of establishing a pregnancy when two or three pre-embryos are replaced is about 15-20% for each procedure. Since PID should still be considered as an experimental method, 106 107 couples using PID are offered verification of the diagnosis by prenatal diagnosis. Liu et al 111 analysed exon 17 in four embryos at risk for a deletion of exons 3-18 and three were found to be normal and were replaced. A singleton pregnancy was the eventual result.

Discussion
Although the possibilities for detecting microlesions in the dystrophin gene are increasing, still for the near future in about 30% of families mutation detection will not be in the standard package of most diagnostic laboratories. Fortunately, linkage analysis and determination of serum CK activities in female relatives mostly enable a fairly accurate determination of carrier risks in these families. This may even be possible when no DNA from the patient is available. However, families with patients who have already died might pose serious problems in assigning carriers risks, although the PTT and other approaches might directly detect female carriers. Therefore, every physician involved with muscular dystrophy patients should obtain and store cells from DMD and BMD patients for DNA and RNA extraction or for establishing a cell line or both.

In practice, DGGE may detect more mutations than SSCP. Therefore, further development of DGGE will be worthwhile. In a two dimensional DNA electrophoresis method, DGGE preceded by extensive PCR multiplexing has recently been proven to be very effective in detecting mutations in the 200 kb sized RB1 gene with its 27 exons. 112 The ideal setting for the future is a quick reliable mutation scanning system for the whole dystrophin gene. Two dimensional DNA electrophoresis may 112-116 might have this potential and needs to be evaluated further.

At present prenatal diagnosis by chorion vilus sampling will be the only realistic option for many DMD and BMD carriers who want to have children of their own. PID needs to be optimised in order to become a more acceptable option for many parents. In polar body biopsy the at risk haplotype or mutation in the polar body implies presence of the normal allele in the primary oocyte, but this method has not been used in diagnosis of DMD or BMD.

As the majority of DMD and BMD cases cannot be prevented, hopefully gene therapy will provide young DMD and BMD patients with a way of stopping disease progression. However, there are still major obstacles to be overcome. 115 116

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