Analysis of five Duchenne muscular dystrophy exons and gender determination using conventional duplex polymerase chain reaction on single cells

Nicole D. Hussey1,6, Hu Donggui1,3, David A.H. Froiland1, Damian J. Hussey2, Eric A. Haan4, Colin D. Matthews1 and Jamie E. Craig5

1Department of Obstetrics and Gynaecology and 2Department of Medicine, University of Adelaide, The Queen Elizabeth Hospital, Woodville 5011, South Australia, Australia, 3Institute of Obstetrics and Gynaecology, The 2nd People’s Hospital, Guangzhou, 510150, People’s Republic of China and 4South Australian Clinical Genetics Service, The Women’s and Children’s Hospital, North Adelaide, 5006, South Australia

5Present address: Centre for Eye Research Australia, Department of Ophthalmology, University of Melbourne, East Melbourne, 3002, Victoria, Australia

6To whom correspondence should be addressed

We have developed five conventional duplex polymerase chain reaction (PCR) protocols on single lymphocytes and blastomeres from embryos, in order to analyse five exons commonly deleted in deletion-type Duchenne muscular dystrophy (DMD). The five DMD gene exons (17, 19, 44, 45 and 48) can be analysed in separate duplex PCR reactions together with the sex-determining region Y (SRY) gene which enables simultaneous gender assignment. We present here PCR amplification results from single lymphocytes isolated from a normal male (220 cells), a normal female (24 cells) and a male DMD patient (40 cells) carrying a deletion of exons 46–49 within the DMD gene. The method failed to produce a PCR signal for the SRY gene in 8/220 normal male cells (3.6%) and for a DMD exon in 0–4.5% of normal male cells. One negative control out of 112 was positive. When this method was used to analyse two blastomeres from each of five embryos, concordant results were obtained for each pair of blastomeres. All embryos produced signals for the DMD exon tested with four of the embryos found to be male and one female. This method is therefore suitable for preimplantation genetic diagnosis and will allow the transfer of healthy embryos (both male and female) in families carrying DMD gene deletions involving at least one of the five exons 17, 19, 44, 45 and 48.

Key words: DMD gene/Duchenne muscular dystrophy/preimplantation genetic diagnosis/single cell PCR

Introduction
Duchenne or Becker muscular dystrophy (DMD/BMD) is one of the most common X-linked lethal genetic diseases with a worldwide frequency of one in 3500 live male births (Harper, 1989). Since no effective therapy exists thus far, most patients die at ~20 years of age. Mutations in the DMD gene can be divided into three different categories of deletions, duplications and point mutations. Deletions within the 79 exon DMD gene account for ~60% of all DMD cases, 98% of which can be detected by two sets of multiplex polymerase chain reaction (PCR) reactions (Beggs et al., 1990; Chamberlain et al., 1990). Prenatal diagnosis using these two multiplex PCR protocols can determine whether a male pregnancy is affected when the deletion mutation for the family is known (Abbs, 1996).

Preimplantation genetic diagnosis (PGD) is a new alternative to conventional prenatal diagnosis particularly for those couples for whom termination of pregnancy is not acceptable. PGD is currently available for a wide range of single gene disorders including many X-linked disorders, cystic fibrosis, and β-thalassaemia (Handyside et al., 1989, 1992; Cui et al., 1995; Coonen et al., 1996; Ray et al., 1996a; Kuliev et al., 1998).

Sexing of embryos for PGD (Handyside et al., 1989) has allowed the transfer of healthy female embryos where embryos are at risk of X-linked diseases such as DMD. However, with a gender-only selection strategy, all male embryos will be discarded even though half of these are not affected and female embryos are transferred regardless of carrier status.

Attempts to develop detection methods for deletion-type mutations that would allow the transfer of healthy male embryos are complicated by the large range of exon deletions present in individual families. A method using primer-extension-preamplification (PEP) followed by a heminested PCR-based assay for specific DMD exons was developed by Kristjansson et al. (1994). These authors reported the amplification of the 5 DMD exons 4, 8, 12, 45 and 48 as well as ZFX/ZFY for gender assignment from single lymphocytes and blastomeres. There are, however, no reports of this technique being applied clinically. Liu et al. (1995) developed a conventional nested PCR approach to specifically amplify exon 17 for PGD for a family carrying a deletion of this exon. They reported a healthy female pregnancy; however, this method can only be used for those families with an exon 17 deletion, representing ~9.4% of all DMD gene deletions (Chamberlain et al., 1988). A protocol for the amplification of three short tandem repeat (STR) loci and exon 50 from the DMD gene has been reported but gave low amplification rates (67–81%) from single buccal cells (Holding et al., 1993). More recently a protocol for detecting linkage analysis in single cells was
used for PGD of embryos from a female who carried a non-
deletion-type DMD mutation (Lee et al., 1998).

In order to identify healthy male embryos we have developed
five conventional duplex PCR reactions to amplify five
individual exons of the DMD gene (exon 17, 19, 44, 45 or 48)
together with the SRY gene for gender determination. The total
time for analysis is ~6.5 h which is compatible with a fresh
embryo transfer on the same day of biopsy. These five duplex
protocols can be used for PGD in families that carry a deletion
of one or more of the exons 17, 19, 44, 45 and 48 to enable
the transfer of healthy male embryos as well as healthy female
embryos.

Materials and methods

**DMD patient**

Genetic material relating to the DMD family and the proband
male involved in this study were provided by the Department
of Cytogenetics and Molecular Genetics at the Women’s and Children’s
Hospital, Adelaide. The deletion mutation involved the DMD gene
exons 46–49 as found by conventional DMD multiplex PCR analysis
from the proband’s genomic DNA. Haplotype analysis by STR44,
STR45 and STR49 linkage analysis revealed that the mother of the
proband was a carrier for the deletion mutation.

**Isolation and preparation of single lymphocytes**

PCR amplifications were performed on single peripheral blood
lymphocytes isolated from whole blood by Lympho-Paque preparation.
Lymphocyte pellets were washed three times in 1× phosphate-
buffered saline (PBS) (150 mmol/l NaCl, 16 mmol/l Na₂HPO₄,
4 mmol/l NaH₂PO₄, pH 7.3) and resuspended in autologous plasma.
A small volume of the lymphocyte mixture was diluted into a pool
of Roswell Park Memorial Institute (RPMI) medium on a glass slide.

**Cryopreservation of lymphocytes**

Cryopreservation allows patient lymphocyte samples to be stored for
up to a year before thawing ampoules for single cell sorting.
Lymphocytes are best isolated from fresh whole blood. After washing
time in 1× PBS and resuspension in autologous plasma,
concentrated lymphocytes (20 µl) were added to 40 µl of fetal calf
serum, 120 µl of RPMI medium and 20 µl of dimethyl sulphoxide
before cryopreservation in liquid nitrogen until required. After thaw-
ing, lymphocytes were washed three times in RPMI medium before
use in single cell sorting.

**Embryo biopsy and isolation of single blastomeres**

Embryo biopsy was performed on frozen–thawed embryos cultured
overnight until they reached the 6–10-cell stage. This work was
approved by the relevant ethics committees for embryos donated to
research. The hole in the zona pellucida was made using a Fertilase™
system (Medical Technologies Montreux, Switzerland). A 30 µm
biopsy pipette was inserted through the hole and a nucleated blasto-
mere was removed. The blastomere was washed three times in
1× PCR buffer (50 mmol/l KCl, 10 mmol/l Tris–HCl, pH 8.3)
—20°C until use. Lymphocytes were washed three times in RPMI medium before
use in single cell sorting.

First round duplex PCR procedure

Two pairs of outer primers, one pair for the DMD locus (either exon
17, 19, 44, 45 or 48) and the other for the SRY gene (Table I) were
used in the first round PCR reaction. To the lysed and neutralized
for each wash and placed into the bottom of a 0.5 ml PCR tube. This
PCR amplifications were performed on single peripheral blood
40 µl of dimethyl sulphoxide (DMSO) containing 0.01% PVP using a freshly pulled Pasteur pipette.

Lysis was carried out by the method of Cui et al. (1989), with 5 µl
of lysis buffer (200 mmol/l KOH, 50 mmol/l dithiothreitol) for 10 min
at 65°C followed by neutralization with 5 µl of neutralization buffer
(300 mmol/l KCl, 900 mmol/l Tris–HCl, pH 8.3, 200 mmol/l
HCl). Negative and positive controls were also lysed. Lysates were
centrifuged briefly and immediately placed back on ice or frozen at
–20°C until use.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Orientation</th>
<th>Primer sequence</th>
<th>Product (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>17 F</td>
<td>GACCTTCGATGTTGACATTTTCCC</td>
<td>416</td>
<td>Chamberlain et al. (1990)</td>
<td></td>
</tr>
<tr>
<td>17 R</td>
<td>AAGCTTGAGATGCCTCTACCTTCCC</td>
<td>416</td>
<td>Chamberlain et al. (1990)</td>
<td></td>
</tr>
<tr>
<td>19 F▲</td>
<td>GATGGCAAAAAATGTTAGAAMAGAGT</td>
<td>459</td>
<td>Chamberlain et al. (1990)</td>
<td></td>
</tr>
<tr>
<td>19 R▲</td>
<td>TCTCACCACATCCCATTTTCTCTCA</td>
<td>459</td>
<td>Chamberlain et al. (1990)</td>
<td></td>
</tr>
<tr>
<td>44 F</td>
<td>GCCCACTTCATTTAAATACGC</td>
<td>497</td>
<td>This paper</td>
<td></td>
</tr>
<tr>
<td>44 R</td>
<td>GACACAACAGCTCAAAGTAATTCC</td>
<td>547</td>
<td>Chamberlain et al. (1990)</td>
<td></td>
</tr>
<tr>
<td>45 F</td>
<td>CATCTCTTATGAGATCTG</td>
<td>506</td>
<td>Chamberlain et al. (1990)</td>
<td></td>
</tr>
<tr>
<td>45 R</td>
<td>TTCAATACATTGGTATAAAACCAACATG</td>
<td>506</td>
<td>Chamberlain et al. (1990)</td>
<td></td>
</tr>
<tr>
<td>48 F</td>
<td>AACAATGGAAGACATCTTGITTGGGGAAC</td>
<td>351</td>
<td>Cui et al. (1994)</td>
<td></td>
</tr>
<tr>
<td>48 R</td>
<td>AAACATGGAACATCCTTGTGGGGAC</td>
<td>351</td>
<td>Cui et al. (1994)</td>
<td></td>
</tr>
<tr>
<td>SRY F</td>
<td>TGTGTCGTTGCATCTCCTCCGCA</td>
<td>351</td>
<td>Cui et al. (1994)</td>
<td></td>
</tr>
<tr>
<td>SRY R</td>
<td>CAGGTTGAAACGGGACAACAGT</td>
<td>351</td>
<td>Cui et al. (1994)</td>
<td></td>
</tr>
</tbody>
</table>

▲The published orientation for these primers (Chamberlain et al., 1988, 1990) is opposite to that given here as we have designated the orientation with respect to the genomic DNA sequence in Chamberlain et al. (1988).
Table II. Sequence of primers used in the second round individual polymerase chain reaction reactions

<table>
<thead>
<tr>
<th>Locus</th>
<th>Orientation</th>
<th>Primer sequence</th>
<th>Product (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>F</td>
<td>GCTGTCACCACCACCTCAGGCACTCA</td>
<td>154</td>
<td>Gelfi et al. (1995)</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CAGAATCCACGATACCTCTTGCACTGCA</td>
<td>313</td>
<td>This paper</td>
</tr>
<tr>
<td>19</td>
<td>F</td>
<td>GAATTACTCATCTTTGCTCTCATGCTC</td>
<td>313</td>
<td>Chamberlain et al. (1990)</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CAATGAACTCAAAGTTGAATTTCTCC</td>
<td>268</td>
<td>This paper</td>
</tr>
<tr>
<td>44</td>
<td>F</td>
<td>CTTCGATCCATATTGCTTTACCTGCAAC</td>
<td>268</td>
<td>Chamberlain et al. (1990)</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>TCCATCACCCTTCAGAACCTGATCTTCC</td>
<td>387</td>
<td>This paper</td>
</tr>
<tr>
<td>45</td>
<td>F</td>
<td>AAAAATGAGCTAACCGAGAG</td>
<td>387</td>
<td>Kristjansson et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CTGTTTGCAGACCTCCTGCCACCACT</td>
<td>308</td>
<td>This paper</td>
</tr>
<tr>
<td>48</td>
<td>F</td>
<td>GCTTATGCCTTGAGAATTATTTACCC</td>
<td>308</td>
<td>This paper</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>AATGAGAAAATTCAGTGATATTGCC</td>
<td>308</td>
<td>Kristjansson et al. (1994)</td>
</tr>
<tr>
<td>SRY</td>
<td>F</td>
<td>CATGAACGCATTCATCGTGTGGTCAC</td>
<td>254</td>
<td>Cui et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CTGCGGGAAGCAAACTGCAATTCTAC</td>
<td>254</td>
<td>Cui et al. (1994)</td>
</tr>
</tbody>
</table>

Cycling conditions were 94°C for 45 s, 56°C for 60 s, 72°C for 60 s for 30 cycles followed by a final extension step of 72°C for 5 min.

Second round PCR procedure

Separate second round PCR reactions were performed for each locus using one pair of inner primers (Table II). A 3.5 µl aliquot of the first round PCR product was used to seed the second round individual PCR reactions. The aliquot was added to 5 µl 10× PCR buffer (500 mmol/l KCl, 100 mmol/l Tris–HCl, pH 8.3), 5 µl 25 mmol/l MgCl₂, 4 µl of 10 mmol/l dNTP mix (2.5 mM each), 10 pmol each of the forward and reverse primers, 1 U Taq polymerase (Perkin Elmer) and ultra pure water (Biotech International) to a volume of 50 µl. After addition of 50 µl of mineral oil tubes were placed into an MJ Research PTC-100 PCR thermal cycler and an initial denaturation step of 96°C for 5 min, cycling conditions were 94°C for 45 s, 52°C for 60 s, 72°C for 60 s for 30 cycles followed by a final extension step of 72°C for 5 min. PCR products were electrophoresed on 2% agarose gels in 0.5× TBE (Tris, borate, EDTA) prestained with ethidium bromide and photographed.

Results

The first round PCR on lysed and neutralized single lymphocytes was performed as a duplex reaction with one set of primers from a DMD exon and the other from the SRY gene (Table I). Individual second round PCR reactions were performed for the DMD exon and the SRY gene on an aliquot of the first round PCR product using one pair of nested PCR primers (Table II).

Using this method we were able to simultaneously amplify a DMD exon with the SRY gene at the single cell level. A total of 220 single lymphocytes from a normal male were amplified using five conventional duplex PCR reactions followed by separate nested PCR reactions. An aliquot of the final wash buffer from the single cell sorting procedure was used as a negative control and three cells from the same normal male utilized as the positive control.

For each duplex reaction the amplification bands for eight single cells are shown (Figure 1) and the total amplification results are given in Table III. PCR product bands for each of the DMD exons and the SRY gene were strong and consistent for all cells and their intensity was similar to the 3-cell positive controls and the genomic DNA controls. The DMD exons amplified in 214/220 cells (see Table III). The amplification failure rate thus varied from zero to 4.5%. Amplification of the SRY gene was satisfactory in 212 of the 220 normal male cells giving an amplification rate of 96.4% and thus a failure rate of 3.6%.

PCR amplification results of single cells sorted after cryopreservation (Figure 1B) were comparable to fresh sorted cells.
Table III. Single cell duplex polymerase chain reaction (PCR) amplification results

<table>
<thead>
<tr>
<th>DMD exon</th>
<th>PCR amplification results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMD exon</td>
</tr>
<tr>
<td>17</td>
<td>42/44</td>
</tr>
<tr>
<td>19a</td>
<td>44/44</td>
</tr>
<tr>
<td>44</td>
<td>42/44</td>
</tr>
<tr>
<td>45</td>
<td>44/44</td>
</tr>
<tr>
<td>48</td>
<td>42/44</td>
</tr>
<tr>
<td>Total</td>
<td>214/220</td>
</tr>
</tbody>
</table>

*Includes results on 20 cryopreserved cells.
*The false positive and false negatives are controls of cryopreserved cells.

Figure 2. Agarose gel electrophoresis of the duplex polymerase chain reactions between an exon of the DMD gene and the SRY gene. Exon 17 (lane 2), exon 19 (lane 3), exon 44 (lane 4), exon 45 (lane 5) and exon 48 (lane 6). The amplification products from single lymphocytes from a normal female are shown. The controls are not shown. pUC19 DNA restricted with HpaII was used as the size standard (lane 1). The sizes of the PCR products are the same as for Figure 1.

Only one false positive was seen during the PCR amplification and this occurred from the wash buffer of cryopreserved cells.

Our primers gave the correct diagnosis in 24 single cells from a normal female. One single cell amplified using each of these duplex reactions is shown in Figure 2. Eight single lymphocytes from an affected male carrying a DMD deletion of exons 46–49 were tested using each of the duplex reactions (Figure 3). The bands observed were consistent with the diagnosis in each case.

The analysis of ten blastomeres removed from embryos at the 6–10-cell stage is shown in Figure 4. Each pair of blastomeres from the same embryo was analysed with the same duplex reaction and concordant results were found. As expected the DMD exon amplified in every case. From this analysis four embryos were found to be male and one embryo was found to be female. Further analysis of additional cells from each embryo and/or an analysis of the biopsied embryo itself to confirm the diagnoses made was not carried out as this is currently prohibited by South Australian legislation.

Discussion

We present here a simple method to duplex one exon of the DMD gene together with the SRY gene for gender determination at the level of a single cell. The clear amplification bands allow confidence in diagnosis. Compared to positive controls the bands from single cells were just as intense, indicating that maximum amplification efficiency was reached for the single cell PCR. A low level of amplification failure was observed for each of the DMD exons varying from zero to 4.5%. Although the amplification failure rate for the pair of SRY primers has been reported to be zero (Cui et al., 1994), we found it to be 3.6%. We confirm that amplification failure is a risk (Ray et al., 1996b; Lissens and Sermon, 1997) for PCR of single cells which can result in misdiagnosis. As shown in Table III the duplex PCR reactions for exon 17, 44 and 48 all showed amplification failure. The failure of one cell to amplify at both exon 48 and the SRY gene (Figure 1e, lane 5) is most likely due to the cell not being placed into the bottom of the tube. Failure to amplify at both loci also occurred on two occasions for the 3-cell positive controls, showing a defect in our technique of single cell sorting. This may be due to the presence of inhibitors, as a larger volume of medium is transferred to the PCR tube when transferring three cells as opposed to transferring a single cell. In five out of 220 single
cells a signal for the SRY gene was produced but not a signal for the DMD gene. Conversely 7/220 single cells produced a band for the DMD gene but not for the SRY gene. We interpret the PCR failures in these cases to be due to the same phenomenon that results in the failure to amplify one allele of a pair of alleles at a heterozygous locus, commonly called allele drop-out (ADO).

The greatest risk of misdiagnosis is the presence of a band for the DMD exon in a male cell when the exon was indeed absent from the blastomere (false positive result). As a measure of our laboratory false positive rate, no amplification of exon 48 was seen for the eight cells from the DMD male that were amplified with the exon 48 duplex reaction. More importantly, none of the negative controls for the blastomere washing procedures produced PCR bands. In addition, none of the 102 negative controls for the sorting of fresh lymphocytes produced false positive results. However, the risk of contamination is always present and therefore couples must be counselled for this small risk of misdiagnosis.

Out of the 112 negative controls for the cell sorting of lymphocytes, one produced a signal and this was from the wash buffer of cryopreserved cells. This is most likely due to the increased difficulty of sorting cryopreserved cells and the increased risk that cells lyse and release their DNA into the surrounding wash medium. No contamination was seen in the 102 negative controls from the sorting of fresh cells. Even though it is more difficult to sort single cells from cryopreserved lymphocyte preparations, cryopreservation has the enormous advantage in that numerous lymphocyte preparations of various genotypes can be stored and used for single cell sorting at a later date.

This study aimed to develop PCR conditions to amplify some commonly deleted DMD exons from single cells for the clinical application of PGD for DMD deletion-type mutation families. We selected the five commonly deleted DMD exons (17, 19, 44, 45, 48) of the 6-multiplex PCR reaction by Chamberlain et al. (1988) as the primers for these exons produce excellent results on genomic, CVS and amniocentesis DNA. Single cell PCR is technically demanding and only well-designed nested pairs of primers are suitable. We tried to use the primers of Chamberlain et al. (1988) as the outer primers and design new pairs of inner primers. However, the exon 44 PCR product of Chamberlain et al. (1988) is very small and contains an unfavourable DNA sequence with which to design primers. Therefore, we used these exon 44 primers as inner primers and designed a new pair of primers as outer primers. For exon 17 we found the published primers of Gelfi et al. (1995) as inner primers to be very good. We also investigated the published primers of Kristjansson et al. (1994) and Liu et al. (1995) as inner primers but found better results with the primers we designed (Table II) except for the inner reverse primer for exon 48 which was used and published by Kristjansson et al. (1994). In this study we have shown that the combination of previously published and newly designed primers work very well for all five duplex PCR reactions on single cells.

Fluorescence in-situ hybridization is used widely for the determination of gender for PGD; however, a PCR method such as that described here is the method of choice when information about single gene disorders from a single blastomere from each embryo is sought. The use of ZFX/ ZFY primers which amplify regions on the X and Y chromosome that can be distinguished using restriction enzyme digestion has been shown to produce misdiagnosis of gender on single cells (Kristjansson et al., 1994). Our SRY results compare favourably with the Y repeat analysis (Handyside et al., 1989) shown to amplify in 92% of normal male cells by Holding et al. (1993).

We have tested the five duplex PCR reactions on blastomeres obtained from embryos donated to research and found concordant results from blastomeres isolated from the same embryo. Therefore we have confidence in offering this method clinically for the preimplantation diagnosis of DMD deletion-type mutations. The combination of deletion detection with gender determination allows healthy female and male embryos to be available for transfer. Although for all exons (except exon 44) the second round PCR can also be performed as a duplex reaction (results not shown), for clarity of diagnosis we recommend it be performed as two separate PCR reactions. This avoids confusion due to carryover of the first round primers into the second round. As an embryo would only be transferred if a band for the DMD exon was obtained, this means that contamination would pose the greatest risk for misdiagnosis. In the absence of external contamination the low level of amplification failure could have only a small risk of misdiagnosis of gender but would not lead to the transfer of an affected male embryo.

Acknowledgements
We wish to thank Michael Barry for performing the embryo biopsies and Rob Richards and Kathy Friend from the Women’s and Children’s Hospital, Adelaide, Australia, for their generous gifts of primers and genomic DNA. This work was supported by an NH & MRC grant to J.E.C. and C.D.M.

References
N.D. Hussey et al.


Received on June 11, 1999; accepted on August 16, 1999