Birth of healthy female twins after preimplantation genetic diagnosis of cystic fibrosis combined with gender determination

Pierre F. Ray¹, Nelly Frydman³, Tania Attié¹, Samir Hamamah³, Violaine Kerbrat², Gérard Tachdjian³, Serge Romana¹, Michel Vekemans¹, René Frydman² and Arnold Munnich¹

¹Département de Génétique, U393, IRNEM, Hôpital Necker Enfants Malades, 75743 Paris Cedex 15, ²Service de Gynécologie-Obstétrique and ³Service d’histologie Embryo-Cytogénétique de la Reproduction, Hôpital Antoine Béclère, 92141 Clamart, France

To whom correspondence should be addressed. E-mail: ray@necker.fr

Two healthy sisters with a familial history of mental retardation were referred to our centre for preimplantation genetic diagnosis (PGD). Their two brothers showed severe mental retardation. The molecular basis for their disorder could not be identified, but one of the sisters and the mother presented a highly skewed pattern of X-inactivation reinforcing the likelihood of an X-linked mode of inheritance. Both sisters requested PGD to avoid the abortion of potentially affected male fetuses. PGD for sex by fluorescent in-situ hybridization was carried out for the first sister and resulted in the birth of a female child. The second sister and her partner, whose niece had cystic fibrosis (CF), were tested for CF mutations, and were both found to be ΔF508 heterozygous. We developed an efficient single cell PCR protocol for the simultaneous amplification of the CF (ΔF508) locus as well as the X-linked amelogenin gene and its highly homologous pseudogene on the Y chromosome. Two PGD cycles were carried out to screen against male and ΔF508 homozygous deleted embryos. In each case several embryos could be selected for transfer and the second cycle resulted in a twin pregnancy followed by the birth of two healthy female infants.

Key words: cystic fibrosis/gender determination/mental retardation/polymerase chain reaction/preimplantation genetic diagnosis

Introduction

Current methods of prenatal diagnosis for chromosomal and single gene defects involve sampling fetal cells by amniocentesis or chorion villus sampling and use of cyogenetic, biochemical or DNA methods to detect the genetic defect. Preimplantation genetic diagnosis (PGD) allows only unaffected embryos to be selected for transfer to the uterus (Handyside and Delhanty, 1997). Any pregnancy should therefore be unaffected and the possibility of a termination at later stages of pregnancy is avoided. For many couples, this is a more acceptable solution than the termination of an established pregnancy. PGD first consisted of the selection of female embryos for patients at risk of transmitting X-linked recessive diseases (Handyside et al., 1989). Specific single cell amplification protocols were then developed for single gene defects, starting with cystic fibrosis (CF) due to the high prevalence of the disease, its severity and its relative molecular homogeneity (Coutelle et al., 1989). CF is the most common severe autosomal recessive disease in the Caucasian population and affects one in 2500 births with a corresponding carrier frequency of about one in 25 (Boat et al., 1989). The predominant defect, accounting for 70–75% of all CF chromosomes, is a 3 bp deletion resulting in the absence of a phenylalanine residue at position 508 in the polypeptide (ΔF508). Lesko et al. developed an efficient nested PCR single cell protocol for ΔF508 diagnosis followed by detection of the deletion by heteroduplex formation (Lesko et al., 1991). Using this method, a first clinical trial of three cycles resulted in the first normal birth following PGD for a single gene defect (Handyside et al., 1992). Other protocols were described and used to successfully treat a couple in which the male had congenital absence of the vas deferens requiring microsurgical aspiration of epididymal sperm and ICSI (Liu et al., 1992, 1994). Avner et al. described the simultaneous detection of the two most common mutations in the Jewish population, W1282 and ΔF508, by multiplex amplification with the outer primers and separate amplification with the inner primers (Avner et al., 1994). Multiplex fluorescent protocols were then described for direct (Findlay et al., 1995) and indirect diagnoses (Dreessen et al., 2000) and numerous clinical cases have been described in the literature (Ao et al., 1996; Goossens et al., 2000).

X-linked mental retardation is also frequent and affects in excess of 1 out of 600 boys (Herbst and Miller, 1980). Two-thirds of these patients remain hitherto uncharacterized, therefore preventing the possibility of specific diagnoses. When the origin of the disorder is truly genetic and X-linked, the boys have a one in two chance of being unaffected. In some other cases, the diagnosis of X-linked inheritance might not be correct and the chances of transmission to a male offspring are then much reduced. In case of a male pregnancy, females at risk of transmitting such a condition have to decide whether or not to abort a fetus that has a 50% chance of being unaffected.

Here we describe a test combining sex determination and ΔF508 detection for a couple at risk of transmitting both a severe X-linked
mental retardation and CF. Due to the high frequency of these two groups of pathologies, we can estimate that the number of couples at risk of transmitting both CF and X-linked mental retardation is similar to that of couples at risk of rare single gene defects. We used a PCR protocol allowing the reliable amplification of the ΔF508 allele as well as the amelogenin gene on the X chromosome and its pseudogene on the Y chromosome. The assay was validated on 94 single lymphocytes and used for PGD. Following the second PGD cycle, a twin pregnancy was achieved. The sex of each twin was confirmed by an ultrasound scan, but the parents did not wish to undergo prenatal diagnosis to obtain confirmation of the CF status. Both twins were typed at birth and one was found to be homozygous wild type and the other heterozygous at the ΔF508 locus. Incidentally, the sister successfully underwent fluorescence in-situ hybridization (FISH) sexing PGD in our unit and delivered a healthy baby girl after two PGD cycles.

**Materials and methods**

**Patients**

Two healthy sisters (II:1 and II:4) with a familial history of mental retardation were referred to our centre (Figure 1). Their mother (I:2) had initiated 11 pregnancies, seven of which resulted in early miscarriages, and the other four in the births of our two patients and of two boys (II:2, II:3) showing severe mental retardation. Both brothers have a normal karyotype and no expansion at the FRM1 locus. They are both placed in specialized institution and present autistic behaviour with no language acquisition. Due to the large number of loci implicated in mental retardation, the molecular characterization or even the localization of such non-syndromic mental retardation is extremely difficult so no attempts were made to find a gene localization in this family (Chelly and Mandel, 2001; review). Levels of inactivation of the X chromosomes for the mother and the two sisters were measured on the basis that female carriers of X-linked defects frequently present a severe bias in their X-inactivation profile. Levels of X-inactivation were measured at the human receptor locus as previously described (Allen et al., 1992). Skewed X-inactivation is usually attributed to the negative selection of the cells expressing the mutant X (Belmont, 1996) and has also been described in female obligate carriers of X-linked mental retardation (Raynaud et al., 2000). Individual II:1 and her mother I:2 presented a highly skewed pattern of X-inactivation, therefore reinforcing the likelihood of an X-linked mode of inheritance. However, this data could not exclude the risk of transmission from her sister I:4 who did not present the skew.

Termination of a male fetus had been carried out for II:1. Subsequently, both sisters (II:1, II:4) requested PGD to avoid the abortion of male fetuses of unknown status. In the course of the genetic counselling it was noticed that II:1’s husband had a niece affected with CF. II:1 and her husband were therefore tested for the most common CF mutations and were both found to be ΔF508 carriers. II:4 was also tested and found to be a ΔF508 heterozygote, but none of the 30 most common CF mutations in the Caucasian population were detected in her husband. PGD sexing by FISH was carried out for individual II:4 and a single cell PCR protocol was developed and applied to II:1 to determine both the sex and the ΔF508 status of each embryo.

**Lymphocyte preparation**

Lymphocytes were separated by centrifugation through Ficoll-paque (Amersham Biosciences, Saclay, Orsay, France) from 10 ml of blood unclotted with citrate-dextrose anti-coagulant (ACD) following the manufacturer’s protocol. Lymphocytes were tubed in a positive pressure clean laboratory (over-shoes, gloves and gowns worn at all times) with access restricted to a small number of people. No DNA work was performed in this room except the assembling of the reagents for PCR. Lymphocytes were handled with a mouth-controlled fine heat-polished glass micropipette in drops of RPMI supplemented with 10% fetal calf serum. The cells were selected and retrieved individually under visual control through an inverted microscope and the whole of the aspirated volume containing the cell (0.1–0.3 μl) was deposited in 3 μl of lysis buffer (200 mmol/l KOH, 50 mmol/l dithiothreitol). The lysis was then completed by a 10 min incubation at 65°C (Cui et al., 1989).

**Amplification of the CFTR exon 10 and the amelogenin genes.**

Prime amplifying the ΔF508 locus were designed using the software Oligo 6.0, to amplify as small a fragment as possible in order to be able to differentiate between the wild type and the mutant alleles (differing by 3 bp) on a simple polyacrylamide minigel. Sequences of the primers, melting temperatures and sizes of the amplified fragments are reported in Table I. The primers for the X-linked amelogenin gene and its highly homologous pseudogene on the Y chromosome were chosen so that there was 100% sequence homology in the X and Y primer sequences. These primers encompass a 5 bp insertion on the Y chromosome and have been previously reported (Ray et al., 2001). This small size difference is ideal as it is big enough to allow easy discrimination between the two bands on a polyacrylamide gel, but is sufficiently small to prevent a size-driven preferential allele amplification.

The first round PCR reaction contained 3 μl of lysis buffer, 3 μl of potassium-free PCR buffer (25 mmol/l MgCl2, gelatine 1 mg/ml, 100 mmol/l Tris–HCl, pH 8.3), 3 μl of neutralizing buffer (900 mmol/l Tris–HCl, pH 8.3, 300 mmol/l KCl, 200 mmol/l HCl) as previously described (Cui et al., 1989; Li et al., 1991), 3 μl of 2 mmol/l dNTP mix (Pharmacia), 3 μl of primer mix containing 5 μmol/l of the outer CF primers and 2.5 μmol/l of the outer amelogenin primers (Genset, Paris, France, see Table I), 0.3 μl of AmpliQ Gold (Perkin-Elmer) and double-distilled water up to a final volume of 30 μl, covered with 30 μl of mineral oil. A total of 3 μl of the first amplification product was transferred to 27 μl of a second round amplification mix which contained 3 μl of Perkin-Elmer PCR buffer (500 mmol/l KCl, 100 mmol/l Tris–HCl, pH 8.3, 15 mmol/l MgCl2), 3 μl of 2 mmol/l dNTP, 2.25 μl of glycerol, 3 μl of primer mix containing 10 μmol/l of the inner CF primers and 5 μmol/l of the outer amelogenin primers and 0.3 μl of AmpliQ Gold.

Denaturation and annealing were carried out successively at 96°C for 20 s and 60°C for 45 s followed by an extension phase at 72°C for 90 s for both the outer and inner reactions. The denaturation temperature of the first 10 cycles of the outer reaction was increased to 98°C to reduce the allele dropout (ADO) rate (Ray and Handyside, 1996). Twenty-two and 26 thermal cycles were carried out for the first and second round reactions respectively in a Perkin-Elmer 9700 thermal cycler. Both reactions were preceded by a 10 min
denaturation at 95°C (to activate the Ampligold) and terminated with a 7 min final extension at 72°C. Amplified products (10 μl) were electrophoresed on 8% polyacrylamide minigels for 60 min at 30 V/cm. The gels were then stained in 0.5 μg/ml ethidium bromide for 5 min, rinsed and exposed to 254 nm UV light for visualization (Figure 2).

The handling of all the reagents and the preparation of the PCR mixes was carried out in a class II cabinet wearing clean gowns and gloves. No contact with the PCR mixes was allowed and all the reagents and the preparation of the PCR mixes was carried out in a separate class II cabinet with filters (Millipore filters) and aliquoted into sterile tubes. Numerous control blanks containing an aliquot of the cell containing media only were also prepared and analysed (see Results).

Clinical embryology, embryo biopsy and cell sampling

Embryo biopsies were performed in the morning of day 3 post-insemination as described previously (Handyside et al., 1989), but using a non-contact 1.48 μm diode laser system (MTM Medical Technology, Montreux SA, Switzerland) for the piercing of the zona pellucida. Two or three short pulses (16 ms) were applied at the intersection of two blastomeres where the distance between the zona and the piercing of the zona pellucida. Two or three short pulses (16 ms) were applied. Gentle negative pressure was applied in the biopsy pipette to withdraw the any blastomere was greatest to minimize potential damage to the adjacent cells. Two or three short pulses (16 ms) were applied diode laser system (MTM Medical Technology, Montreux SA, Switzerland) for

Table I. Primer sequences, melting temperatures (Tm) and sizes of amplified products

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Tm (°C)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cystic Fibrosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outer/inner forward</td>
<td>TCTGTTCGTCTCGTTTCTCTGATTA</td>
<td>70.2</td>
<td>89</td>
</tr>
<tr>
<td>Outer reverse</td>
<td>TATTGGGTTGTTGAGGGGTCTAT</td>
<td>71.2</td>
<td>177</td>
</tr>
<tr>
<td>Inner reverse</td>
<td>ATGCTTTGAGAGCGCTCTGTAACTA</td>
<td>70.8</td>
<td>102</td>
</tr>
<tr>
<td>Amelogenin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outer forward</td>
<td>TGGGCACCCCGTGGTATTTCAACT</td>
<td>72.1</td>
<td>207</td>
</tr>
<tr>
<td>Outer/inner reverse</td>
<td>AGGCAAAACATCAAGCTTAAACT</td>
<td>71.9</td>
<td>207</td>
</tr>
<tr>
<td>Inner forward</td>
<td>CCCCTGGCTCTGTAAAAGATAGTG</td>
<td>70.7</td>
<td>120</td>
</tr>
</tbody>
</table>

When ≥3 unaffected embryos were suitable for transfer, embryos with the best morphology were selected for transfer. To avoid late evening transfers, uterine replacement of unaffected embryos were carried out in the morning of day 4 post-insemination, as has been previously implemented (Grifo et al., 1998).

Results

Amplification of single lymphocytes

Accuracy and efficiency of the diagnosis were tested on lymphocytes of both sexes and of different genotypes. Emphasis was put on the analysis of heterozygotes to determine ADO rates. In total, 94 lymphocytes were analysed. Only two failed to give a signal altogether (98% amplification rate). Twenty-eight control blanks were analysed and there were two occurrences of contamination. In one instance, the contamination consisted of the wild-type CF fragment and in the other, it was the X amelogenin fragment. Considering the X and Y amelogenin genes as two alleles of a single locus, the contamination cession consisted of the wild-type CF fragment and in the other, it was the X amelogenin fragment. Considering the X and Y amelogenin genes as two alleles of a single locus, the contamination rate for each locus was 1/28 (3.6%).

Among the 94 lymphocytes tested, 13 were male homozygous wild type, five female homozygous wild type, 34 male heterozygous and 42 female heterozygous. One from each of the last two categories did not yield any signal. There were four occurrences of ADO: two absences of amplification of the Y amelogenin in male cells (in one homozygous and one heterozygous cell) and two absences of amplification of the deleted allele in cells heterozygous at the ΔF508 locus (each time in male cells). ADO for the amelogenin was therefore two out of 47 male cells (4.2%) and for the CF locus, two out of 74 heterozygous cells (2.7%).

Cycle 1

Fourteen oocytes were collected, yielding 10 zygotes with two pronuclei after ICSI (Table II). All embryos were biopsied and a...
diagnosis was obtained from all but one (90%). There were two male and seven female embryos.

Among the males one was heterozygous and one was homozygous non-deleted at the ∆F508 locus. There were three heterozygous, one homozygous wild type and three homozygous deleted female embryos. The three heterozygous female embryos (3, 6 and 7) had the best morphology and were selected for transfer. Unfortunately this transfer did not result in a pregnancy. Due to the complexity of the diagnosis, mostly in the non-amplification of the Y band and hence the misdiagnosis of a male embryo, efforts were made to biopsy two cells from most embryos. One cell only was removed from embryo numbers 1 and 2 due to the small number of cells and poor embryo morphology (Table III). A third cell was removed from embryos 9 and 10 due to lysis or absence of the nucleus of some of the biopsied blastomeres. In embryo 10, two cells came out together during the biopsy of the third blastomere; however, only one of these two cells showed a nucleus. Overall 19 blastomeres were analysed along with 13 blanks and two control lymphocytes. Sex and CF status were correctly identified in both control lymphocytes. No amplification was detected in any of the blanks. Results were obtained for at least one locus in 13 cells (68% amplification rate) and for both loci in 11 out of 19 cells (58% amplification rate).

**Cycle 2**

Twenty-eight oocytes were collected, yielding 15 normally fertilized two pronuclear embryos (Tables II and III). All embryos were biopsied and a diagnosis was obtained from all but one (93%). There were six male and eight female embryos. Among the male embryos, there was no homozygous wild type at the ∆F508 locus, three heterozygous and three homozygous ∆F508-deleted embryos. Among the female embryos, there were three homozygous wild type, three heterozygous and two homozygous deleted embryos. Two cells came out together during the biopsy of embryo 4, resulting in the removal and analysis of three cells. In embryo 11, the first blastomere biopsied lysed and the presence of a nucleus was questionable in the second blastomere. A third cell was therefore removed and only that last cell gave a genetic result for this embryo. In total 30 blastomeres were analysed with two control lymphocytes and 16 blanks. Sex and CF status were correctly identified in both control lymphocytes. No amplification was detected in any of the blanks. Results were obtained for at least one locus from 26 blastomeres (87% amplification rate) and for both loci in 25/30 blastomeres (83% amplification rate). Three of the four cells which did not yield any PCR product had a questionable nucleus (not clearly visible under careful visual inspection) and the fourth had lysed. Concordant results were obtained for both loci 10 embryos and we noted one instance of ADO of the CF wild-type allele in one of the cells from embryo 4. PCR results obtained for each cell analysed from the first four embryos are shown in Figure 2. The absence of amplification (ADO) of the deleted CF allele in the second cell of embryo 4 is shown in the penultimate track. All four bands otherwise corresponding to the X, Y, CF and ∆F508 alleles can be seen on the other two cells analysed from embryo 4. The band patterns for a female homozygous deleted (embryo 1), a female homozygous non-deleted (embryo 2) and a female heterozygous (embryo 3) can be seen in the other lanes of the gel.

Due to the good morphology of the embryos, only two embryos were selected for transfer early on day 4 post-insemination. Embryos 5 and 7 were chosen for transfer among the six transferable embryos. Supernumary embryos were kept in culture and three embryos were frozen at the blastocyst stage on day 6.

**Confirmation of diagnosis and pregnancy outcome**

French legislation is still very ambiguous about ‘research’ on human embryos. It can be carried out in some instances, but should be beneficial to the patient and not damage the embryo (Viville and Nisand, 1997). If the confirmation of diagnosis is of benefit to the patient, damage to the embryo seems unavoidable. For this reason reanalysis of the embryos was not carried out.

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**Table III. Summary of the results for the first (cycle 1) and second (cycle 2) CF and sexing PGD. The number of blastomeres in each embryo prior to biopsy and a general indication of their morphology are indicated. Blastomere morphology, in column ‘cell’ indicates whether a nucleus could be distinguished and if the cell had lysed. 0/n corresponds to the visualization (1n) or not (0n) of a nucleus in the biopsied blastomeres.**

<table>
<thead>
<tr>
<th>Number of cells and embryo morphology</th>
<th>Cell</th>
<th>Amplification</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>An. gen. CFTR</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>X  Y  WT  ∆F</td>
<td></td>
</tr>
</tbody>
</table>

**Cycle 1**

| 1) 5 cells, poor | In ? | + + + + | Male non-deleted |
| 2) 4 cells, fair | In ? | + + - + | Female deleted |
| 3) 8 cells, good | In, lysed | - + + + | Female heterozygote |
| 4) 4 cells, fair | In | + + + + | Male heterozygote |
| 5) 7 cells, fair | In | - - - - | Weak amplification, female non-deleted |
| 6) 8 cells, good | In | + - + + | Female heterozygote |
| 7) 8 cells, good | In, lysed | + - + + | Female heterozygote |
| 8) 8 cells, fair | In, lysed | - - - - | No result |
| 9) 8 cells good | In, lysed | + - + + | Female deleted |
| 10) 7 cells, good | In ? | - - - - | Female deleted |

**Cycle 2**

| 1) 6 cells, poor | In | + + - + | Female deleted |
| 2) 7 cells, fair | In | + + - + | Female non-deleted |
| 3) 6 cells, poor | In | + + - + | Female heterozygote |
| 4) 8 cells, fair | In | + + + + | Male heterozygote |
| 5) 8 cells, fair | In | + + + + | Female heterozygote |
| 6) 8 cells, good | In | + + + + | Male deleted |
| 7) 8 cells, good | In | + + - + | Female non-deleted |
| 8) 8 cells, good | In | + + - + | Female deleted |
| 9) 8 cells, good | In | + - - - | Female non-deleted |
| 10) 8 cells, fair | In | + + + + | Male heterozygote |
| 11) 8 cells good, In, lysed | - - - - | Male deleted |
| 12) 8 cells good | In | + + + + | Female heterozygote |
| 13) 6 cells, fair | In ? | - - - - | No result |
| 14) 8 cells, good | In | + + + + | Male heterozygote |
| 15) 7 cells, fair | In ? | + + - + | Male deleted |

Lyset = lysed blastomeres. Plus (+) and minus (−) signs indicate whether a band was observed corresponding to the amelogenin (X) and (Y), the cystic fibrosis wild type (WT) and deleted CFTR (AF) genes. In the diagnosis column, T indicates the embryos chosen for transfer.
For both patients, the sex of the fetuses were confirmed by an ultrasound scan at 16 weeks. Individual II.1 refused prenatal confirmation of the CF status of the twins. DNA was extracted from cord blood of the twins at birth and analysed in our molecular diagnosis laboratory. As established during the preimplantation analysis, one of the twins was heterozygous and the other was homozygous wild type at the ΔF508 locus.

Both pregnancies were uneventful. Patient II:4 delivered at term a healthy 3.30 kg girl. Her sister delivered twin girls of 2.99 and 2.88 kg for 50 and 48.5 cm respectively. The twins were delivered at 37 weeks by Caesarean section due to bad positioning, presently they are both healthy.

**Discussion**

There are two main criteria for a viable diagnostic test. Firstly, it has to be accurate to minimize the chance of misdiagnosis. Secondly, it has to be efficient, as it is necessary to obtain results in the majority of the assays performed. This was particularly important in the present situation as more embryos were likely be discarded due to the double diagnosis of gender and CF. Statistically three-quarters of the female embryos would be homozygous wild type or heterozygous and suitable for transfer. This would correspond to only three embryos out of eight, or 37.5% of the starting embryos.

Here the multiplexing of CF with the amelogenin gene did not increase amplification failure or ADO rates, which are lower than those previously reported at the CF locus (Ray et al., 1996, 1998). We therefore did not feel that the multiplexing diminished in any way the efficiency and accuracy of the test. Previous work showed that efficient co-amplification of six separate dystrophin exons could be obtained with good reliability (Ray et al., 2001).

Contamination by exogenous cells or amplified DNA is one occurrence that can lead to misdiagnosis. No contamination was obtained from the 28 blanks analysed during the tubing of blastomeres during clinical cases tubed in the IVF laboratory, which is completely separate from the molecular laboratory. In contrast, two out of the 28 negative controls analysed during the amplification of lymphocytes amplified one allele from the two loci analysed. The lymphocytes and their negative controls were prepared in the pre-PCR molecular laboratory located close to the post-PCR laboratory in the genetics department. Only one allele from one locus was amplified in each of the contaminated blanks. This therefore suggests that the contaminants were probably single amplicons rather than whole contaminating cells which would more likely give a signal for both loci analysed. This hypothesis is strengthened by the fact that contaminations were only observed in the blanks prepared in the laboratory located closer to the source of contaminating amplicons. Although a relatively small number of negative controls were analysed in this study, the results are in full agreement with that previously observed (Ray et al., 2001) and confirms the importance of using physically separated laboratories for the different steps of the single cell amplification process. Thorough UV decontamination of the laboratory is now carried out weekly to prevent the spread of contamination which could compromise the clinical application of CF diagnosis.

The absence of amplification of one allele during the amplification of single heterozygous cells (ADO) is now well recognized and has been shown to vary from 0–20% in different PCR systems (Ray et al., 1996; Thornhill et al., 2001). ADO of recessive diseases cannot lead to the transfer of affected embryos, but in dominant conditions or in sexing protocols such as the one utilized here, the sole absence of amplification of the mutant allele or of the Y sequence would lead to a serious misdiagnosis. For this reason, here we would only select for transfer embryos for which concordant results had been obtained from two blastomeres at the amelogenin locus. When one of the first two biopsied blastomeres had lysed or did not show a nucleus, a third cell was removed. On two occasions two cells were removed together, probably due to the learning process of the person performing the biopsy. With increased experience we do not tend to observe these occurrences. All blastomeres, lysed and anucleate included, were analysed as previous experience showed that correct results could be obtained from these cells.

In a large study, a total of 484 single blastomeres were analysed in the course of 17 PGD cycles for CF (Ray et al., 1998). Amplification was obtained from 28/39 (72%) lysed blastomeres and 16/45 (35%) anucleate blastomeres. Diagnosis accuracy was reduced in lysed blastomeres to 79 compared with 88% for anucleate blastomeres and 90% for normal nucleate blastomeres. In another study, amplification was obtained from only 10% of blastomeres scored as anucleate by microscopic visual examination (Liu et al., 1993). A case of misdiagnosis was also suspected following the analysis of a lysed blastomere (Sermon et al., 1997). In this study, the blastomere amplification rate was 58% (11/19) and 83% (25/30) for the first and second PGD cycle respectively. The lower amplification efficiency in the first cycle might be related to the lower embryo quality and the higher proportion of lysed and anucleate blastomeres in the first cycle (Table III). The efficiency of lymphocyte amplification (98%) appears higher than that of blastomeres. However, if only nucleated non-lysed blastomeres are included, blastomere amplification efficiency increases to 89% (31/35). This suggests that the lower amplification efficiency observed in blastomeres is probably due to the high proportion of abnormal embryonic cells with compromised DNA, rather than to a factor affecting the amplification process which is intrinsic to the blastomeres.

We feel that diagnoses should not be established on the results from a single lysed or anucleated blastomere, but one can take these results into consideration as confirmation of a diagnosis obtained from another morphologically normal blastomere. For that reason it is worth analysing all biopsied cells, but it is important to aim at two unlysed nucleate blastomeres for each embryo with sufficient cell numbers. At the moment, we do not biopsy only embryos from which two cells can be removed (≥7 cells) as previously suggested (Van de Velde et al., 2000). We might however adopt this measure in the near future as the analysis of cells from poor grade embryos with lowered amplification rate and diagnosis reliability might well prove counterproductive (Ray et al., 1998; Van de Velde, 2000).

In this study, a semi-nested PCR strategy followed by ‘traditional’ gel electrophoresis and ethidium bromide staining were used. Most PGD centres now utilize fluorescent (F)-PCR which is faster and more sensitive (Sermon, 2001). More importantly, it is the finer gel separation power which makes F-PCR an absolute must for PGD as it allows the easy analysis of any polymorphic CA repeat. These polymorphic sites present throughout the genome are ideal tools for the indirect diagnosis of any localized gene (Dreesen et al., 2000; Apessos et al., 2001). By multiplexing the disease locus with flanking markers a double or triple diagnosis is possible, therefore allowing a confirmation of diagnosis from only one analysed cell. We did not choose F-PCR in this study because of practical reasons and limited resources. Nested PCR protocols are robust and relatively easy to set up and allowed us to answer our patients’ demands as quickly as possible. However, we are undergoing the transition from EtBR to fluorescence and are setting up new diagnoses such as myotonic dystrophy and fragile X using F-PCR.

Several couples have been referred to us where the woman is heterozygous for ΔF508 and the man is compound heterozygous with ΔF508 and another CF mutation, often R117H, and presents a bilateral absence of the vas deferens as sole CF manifestation. These couples,
who need ICSI due to male infertility, have a 25% chance of having a child homozygous for ΔF508 with a severe CF phenotype. Compound heterozygotes are not expected to develop more severe symptoms than their father since they have the same genotype. The simplest diagnosis that can be proposed is therefore a straightforward exclusion of ΔF508 homozygotes. Half of the male embryos heterozygous for ΔF508 will however also have inherited R117H and will most probably present a bilateral absence of the vas deferens like their father. To avoid this situation, all ΔF508 carrier embryos could be excluded. According to Mendelian laws this will only leave 25% of the starting embryos. The best practice would be to identify a normal allele in each of the transferred embryos using an indirect diagnosis similar to that proposed by Dreesen et al.; this leaves 50% of the starting embryos and avoids the possibility of transmitting the infertility phenotype (Dreesen et al., 2000). Using our straightforward multiplex test, one could avoid the transfer of male ΔF508 carrier embryos, therefore also leaving a pool of 50% of the starting embryos composed of 25% homozygous wild-type embryos and 25% heterozygous female embryos. Half of the heterozygous female embryos will also have inherited R117H, but should not present any marked phenotype. In practice this is not feasible in France as the absence of the vas deferens would not be considered ‘serious enough’ to perform PGD.

Here we report the successful PGD of two sisters, and in particular one for which a double diagnosis was carried out. French legislation to perform PGD allows couples who have a genuine medical need for this advanced technique. It is the moral obligation of assisted reproduction and genetic counselling professionals to ponder upon the indications and accept only those couples who have a genuine medical need for this advanced diagnostic method.

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References


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