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ARTICLE

Multiplex PCR combining deltaF508 mutation and intragenic microsatellites of the CFTR gene for pre-implantation genetic diagnosis (PGD) of cystic fibrosis

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One major limitation of pre-implantation genetic diagnosis (PGD) practice comes from the need to develop single cell PCR protocols. For a disease such as cystic fibrosis (CF), for which almost 1000 mutations have been identified, the development of a mutation based PGD protocol is impracticable. An elegant way to overcome this problem is to set up an indirect diagnosis using polymorphic markers allowing the identification of the pathogenic haplotype instead of the mutation. We present here a new PGD protocol for CF. Our strategy is based on a multiplex fluorescent PCR co-amplifying the ΔF508 mutation and two CFTR intragenic polymorphic microsatellites (IVS8CA and IVS17bCA). Such an approach is justified since in 91% of the cases at least one partner of the couple carries the ΔF508 mutation. The use of intragenic markers reduces the risk of misdiagnosis due to meiotic recombination. In 97% of the single lymphoblasts (151/155) tested a PCR signal was obtained. A complete haplotyping was achieved in 137/151 (91%) lymphoblasts and a 6% rate of allele drop out (ADO) was observed. Three cases were performed. Case one was at risk of transmitting mutations ΔF508 and R1162X, case 2 ΔF508 and R1066C and case 3 ΔF508 and 1341+1A. Considering these three cases and the re-analysis of the affected embryos, we have analysed 62 blastomeres from which we had PCR signal for 58 (94%) and a complete haplotype for 49 (84%). With the degree of polymorphism of the markers used in this work (48 and 39%) and the fact that we co-amplified the F508 locus our test should be suitable for nearly 80% of the couples requesting PGD for CF. This fluorescent multiplex PCR indirect diagnosis provides also a safer test since it allows the confirmation of the diagnosis, the detection of contamination and could give an indication on the ploidy of the embryos tested.
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Introduction

Pre-implantation genetic diagnosis (PGD) represents an alternative to prenatal diagnosis (PDN) avoiding the burden of a termination of pregnancy (TOP) if the embryo or foetus is diagnosed as affected. PGD requires the combined efforts of geneticists and workers in the field of reproductive medicine. It involves *in vitro* fertilisation (IVF), embryo biopsy to obtain one or two cells from eight-cell stage embryos and genetic analysis of the isolated cells by polymerase chain reaction (PCR) or fluorescent *in situ* hybridisation (FISH). PGD is available for an expanding range of genetic defects such as point mutations, deletions or insertions involved in monogenic diseases (recessive or dominant) or chromosomal

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abnormalities.[1,2] The major difficulty of the PGD procedure relies on the genetic analysis of a single cell. When analysed by PCR this involves the successful amplification of a specific DNA copy of each chromosome. Although the efficiency and the accuracy of the diagnosis procedure has increased with the introduction of fluorescent PCR[3,4] and multiplex PCR,[5,6] a low risk of misdiagnosis persists.[7] Another difficulty is the development of multiplex PCR from a single cell. The range of mutations responsible for a particular disease is another difficulty in the development of new PGD. One way to considerably improve the accuracy and efficiency, while limiting the number of assays to diagnose one specific disorder, would be to base the diagnosis on the characterisation of the pathogenic haplotype (using polymorphic markers) rather than on the identification of the mutation.

Amongst monogenic disorders, cystic fibrosis (CF) represents the most common request for PGD.[2] CF is the most common severe autosomal recessive disorder in the Caucasian population with a carrier frequency of one in 25, affecting one in 2500 newborn children. The major symptoms are obstruction and chronic infection of the respiratory tract and pancreatic insufficiency leading, in most of the cases, to an early death. A minor form of CF results in a congenital bilateral absence of the vas deferens (CBAVD).[8,9] Since CBAVD does not affect spermatogenesis, an IVF with intracytoplasmic sperm injection (ICSI) with testicular sperm can be offered to these patients.[10–12] If the female partner is also carrier of a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, the ICSI procedure can be followed by a PGD. There have been almost 1000 mutations characterised in the CFTR gene (http://www.genet.sickkids.on.ca/cftr/). The mutation ΔF508, (deletion of the phenylalanine at codon 508) accounts for almost 70% of the mutations in the Caucasian population, all the other mutations have a frequency ranging from a few per cent to private mutations. Therefore different situations can be encountered: (i) couples where both partners carry the ΔF508 mutation (49%). In such cases, a simple ΔF508 detection test can be used;[13–15] (ii) couples with one partner carrying the ΔF508 mutation and the other partner carrying another mutation (42%). In this case, a ΔF508 detection test can also be used to eliminate all embryos with the ΔF508 mutation. Such a strategy, even though proposed, is not satisfactory because it results in the rejection of healthy ΔF508 carrier embryos; (iii) couples where both partners carry a mutation different from ΔF508 (9%). In this last situation, either a specific test is developed for each new mutation, which has been done for the most common mutations[16–18] or an indirect diagnosis is set up.[17,19–21] Allele identification combines the following advantages: (i) availability of a single test applicable for a large number of couples as soon as they are informative for the polymorphic markers used; (ii) availability of an internal amplification and contamination control; (iii) the use of two or more polymorphic markers provides additional information, increasing the accuracy of the diagnosis; (iv) to provide an evaluation of the ploidy status of the blastomere tested.[19]

We present here a novel fluorescent multiplex PCR strategy based on the coamplification of the ΔF508 mutation and of two polymorphic intragenic microsatellites, IVS8CA and IVS17bCA. This strategy, albeit similar, differs from the one described by Dreesen *et al*;[19] who used external markers only and did not simultaneously detect the ΔF508 mutation. Such an approach is warranted since in most cases (91%) at least one member of the couple carries the ΔF508 mutation. This strategy allows PGD for informative couples at risk of transmitting CF to their children whatever CF mutation is implied. Furthermore, we report three CF PGD cycles performed in our centre.

Material and methods

Single lymphoblast and blastomere testing

Three Epstein-Barr transformed lymphoblast cell lines were used to perform single cell analysis: one homozygous for the ΔF508 mutation (ΔF/ΔF; line GM04540A; Coriell Cell Repositories, Camden, USA), one heterozygous for this mutation (ΔF/F; line CF carrier, kindly provided by Dr K Sermon, Dutch-speaking Brussels free University, Belgium) and one homozygous normal for the F508 locus (F/F; line SMNt, kindly provided by Dr Judith Melki, Genopole Evry, France). For the three cell lines the marker profiles were first determined on genomic DNA. Tubing procedure was performed as previously described.[15] Supernumerary IVF embryos not suitable for freezing and therefore scheduled for destruction were incubated for 10 min in an embryo biopsy medium, Ca and Mg free (EBM, Medicult-France, Lyon, France) prior to biopsy. They were then biopsied as previously described[22] under an inverted microscope (Leica, Wetzlar, Germany) using Research Instrument micromanipulators and micropipettes (Research Instruments Ltd, Penryn, UK). Zona pellucida drilling was performed using a laser set up (Hamilton-Thorn, Beverly, USA) and blastomeres were gently aspirated and placed in G2.1 medium (Scandinavian Science, Gothenburg, Sweden). Each blastomere was washed three times in EBM, transferred into the reaction tube containing 2.5 μl of Lysis Buffer (LB: 200 mM KOH, 50 mM DTT) and frozen at -20°C until amplification by PCR.

PCR reactions

IVS8CA and IVS17bCA are CFTR intragenic markers with heterozygous frequencies of 48% and 39% respectively.[23,24] Primers for IVS8CA and IVS17bCA amplification were designed to have a similar annealing temperature so that the DNA fragments could be co-amplified with the F508 locus in a single-round of fluorescent multiplex PCR (Table 1). For each primer pair, one was fluorescently labelled with 6-fam (F508) or tet (IVS8CA) or hex (IVS17bCA) fluorochrome (Perkin Elmer Applied Biosystems, Warrington, UK). Aliquots of 22.5 μl PCR master mix containing neutralising buffer

Table 1 Primers used for single cell multiplex PCR

Locus	*Primer name*	*Primer sequence*	*Fluorochrome*
F508	DeltaF508F	GTTTTCCTGGATTATGCCTGGCA	6-fam
	DeltaF508R	GTTGGCATGCTTTGATGACGCTTC	
IVS8CA	AC8F	AAATCTATCTCATGTTAATGCTGAAGA	tet
	AC8R	ACTAAGATATTTGCCCATTATCAAGTT	
IVS17bCA	AC17F	TGTCACCTCTTCATACTCATATTGG	hex
	AC17R	AAACTTACCGACAAGAGGAACTCTG	

(NB: 900 mM TrisHCl, 300 mM KCl and 200 mM HCl), 1.5 mM MgCl2, 200 μM each dNTP, 0.2 μM each primer for F508 locus, 0.4 μM each primer for IVS8CA and IVS17bCA loci and 1.5 U HotStar Taq DNA Polymerase (Qiagen, Courtaboeuf, France) were added to the reaction tubes. PCR reactions were performed using a Mastercycler Gradient PCR machine (Eppendorf, Hamburg, Germany). The program used was 15 min of initial denaturation at 95°C followed by 30 s of denaturation at 96°C for the first 10 cycles and 94°C for the remaining 35 cycles. Annealing and elongation cycles were performed for 30 s at 61°C and 30 s at 72°C, respectively. Reactions were terminated by 10 min at 72°C. PCR products were analysed on either an ABI310 or an ABI377 automated sequencer (Applied Biosystems, Warrington, UK). PCR efficiency was calculated from samples for which a PCR signal for at least one tested locus was obtained.

Informativity testing for couples requesting PGD

Genomic DNA from each couple requesting PGD and from their affected foetus/child was extracted from whole blood samples or from an amniocentesis sample using the standard saline protocol.[25] Haplotype analysis for IVS8CA and IVS17bCA microsatellites and segregation analysis with regard to the locus F508 of the CFTR gene were performed using the triplex PCR procedure described above, except that NB was replaced by buffer II (BII: 500 mM KCL, 100 mM Tris HCl pH 8.3) and 30 cycles were sufficient for amplification.

Clinical PGDs

Three couples requested PGD for CF.

Case 1: a 30-year-old woman and her 26-year-old husband had previously had a TOP after prenatal diagnosis of a foetus diagnosed because of an hyperechogenic bowel detected during ultrasonography. This foetus was a compound heterozygote for mutations ΔF508 (paternally inherited) and R1162X (maternally inherited).

Case 2: a 33-year-old woman and her 34-year-old husband had an affected girl with a severe form of CF. She is a compound heterozygote for mutations ΔF508 (maternally inherited) and R1066C (paternally inherited).

Case 3: a 29-year-old woman and her 33-year-old husband with an affected girl with a severe form of CF had experienced a TOP. The daughter and the affected foetus were compound heterozygotes for mutations ΔF508 (maternally inherited) and 1341+1A (paternally inherited).

For the three couples, ovarian stimulation, oocyte recovery and ICSI procedures were carried out using standard protocols.[26,27] Three days after ICSI, embryos were biopsied and blastomeres were collected – one blastomere from embryos with less than six cells and two from embryos with six or more cells – and analysed. For each biopsied cell, a blank control was prepared from the final EBM wash drop. PCR controls containing single lymphoblast from control cell lines (ΔF/F and ΔF/ΔF) were added.

From untransferred affected embryos at least two blastomeres were individually reanalysed with the exception of embryo 10 from case 2 which was reanalysed as a whole.

Results

Lymphoblast testing

Table 2 shows the results of triplex PCRs performed on single lymphoblasts from three different cell lines. All three lines were informative for both markers tested. We performed 155 single cell analysis for which an amplification of at least one marker was obtained in 151 (97%) single lymphoblasts. Genotypes were reliably determined in 144 out of 151 (95%), in 143 out of 151 (95%) and in 145 out of 151 (96%) of cells for loci F508, IVS8CA and IVS17bCA, respectively. Complete haplotyping was achieved in 137 out of 151 (91%) lymphoblasts. A global allele drop out (ADO) rate of 6% was observed whatever locus and whatever cell line was considered. Eight blanks out of 58 (14%) presented a PCR signal for at least one of the loci tested. Such a contamination rate is quite high, but it is worth noticing that in one experiment five blanks out of 22 presented a PCR signal, accounting for most of the contaminations detected in these experiments. For all the other experiments the contamination rate was acceptable.

Blastomere testing

Thirteen blastomeres from surplus untransferable and unfreezable embryos were tested. Positive amplification was observed in 12 out of 13 (92%) samples. Ten (83%) of them

gave a positive PCR signal for all three loci. The accuracy of the diagnosis was impossible to evaluate since we did not know the parental haplotype, which also prevented the evaluation of the ADO rate. Five out of 13 blank controls showed amplification (38%). Such a high rate of contamination is probably due to the poor sample quality since some blastomeres were lysed during biopsy and DNA might be present in the wash drop as previously noticed.[4,28] It is worth noticing that our contamination rate during the last ten PGD performed in our centre was 6.7% over 89 blanks tested.

We thus concluded that our triplex PCR is efficient and reliable and felt confident for clinical application.

Informativity testing for couples requesting PGD

The results of haplotype analysis are shown in Figure 1. Genotyping was performed in any case on both parents and for case 1 on an amniocentesis sample from an affected foetus (only marker IVS8CA was informative); for case 2 on their affected daughter (only marker IVS8CA was informative); for case 3 on their affected daughter (both markers could be used for the PGD). In all cases a triplex PCR was performed since the non-informative marker can still be used to detect DNA contamination.

Clinical PGDs

The results of PGD cycles are summarized in Table 3. An electrophoregram is shown in Figure 2.

Case 1: Nine cumulus-oocyte complexes were retrieved from which seven were inseminated by ICSI and four 2PN stage embryos were obtained. On day 3, four embryos had reached the eight cells stage and two blastomeres per embryo were biopsied. Three embryos were diagnosed as affected and one (embryo 2) presented a discordant result between the two blastomeres analysed, no embryo was transferred. Reanalysis of at least two blastomeres from the untransferred embryos confirmed our previous diagnoses even for embryo 2, for which a discordant result was again obtained. (Table 3).

Case 2: 20 cumulus-oocyte complexes were retrieved from which 18 were inseminated by ICSI, 14 of which exhibited two pronuclei after ICSI. Three days after fertilisation, 11 embryos were suitable for biopsy and one or two blastomeres were successfully removed and analysed. Nine embryos gave a clear result: one was unaffected, three were carriers and five were affected. A complete amplification failure was obtained for embryo 17. This embryo was not transferred and the re-analysis of two blastomeres diagnosed it as affected. For embryo 18,

Table 2

Cell types	*No. of cells analysed*	*No. of PCR with a signal (%)*	*Signal at the F508 locus (%)**	*Signal at the IVS8CA locus (%)**	*Signal at the IVS17bCA locus (%)**	*Complete genotype (%)**	*ADO (%)*	*No. of blanks*	*Contamination (%)*
ΔF/F	76	74 (97)	71 (96)	71 (96)	70 (95)	68 (92)	6 (8)	24	2 (8)
ΔF/ΔF	51	49 (96)	45 (92)	44 (90)	48 (98)	42 (86)	3 (6)	22	6 (27)
F/F	28	28 (100)	28 (100)	28 (100)	27 (96)	27 (96)	0	12	0
Total	155	151 (97)	144 (95)	143 (95)	145 (96)	137 (91)	9 (6)	58	8 (14)

*Per cent were calculated from sample given a PCR signal for at least one of the tested locus. ADO: Allele drop out.

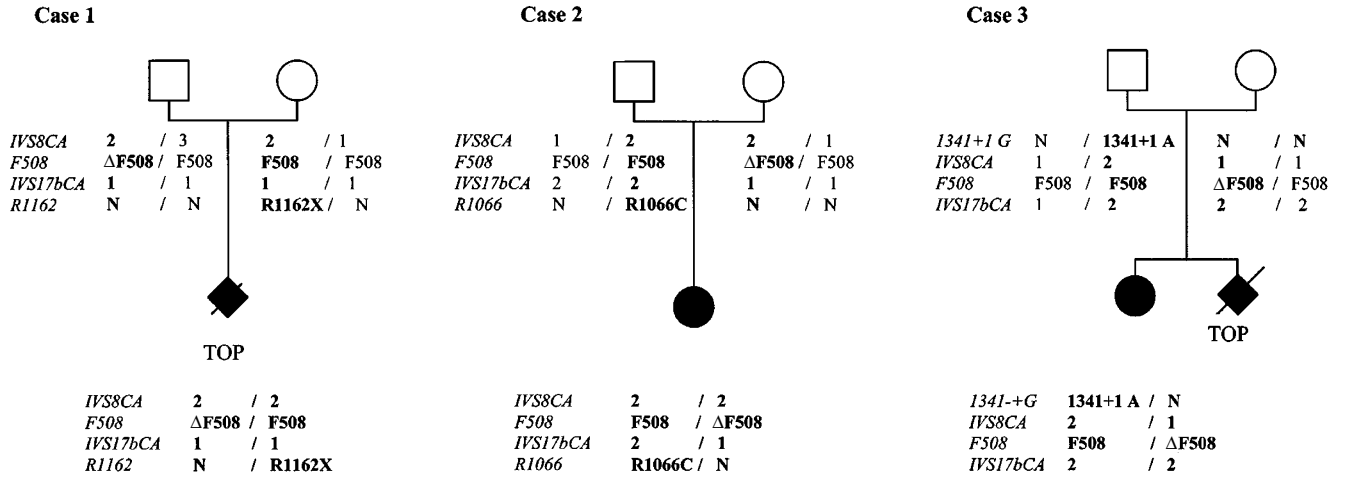

Figure 1 Pedigrees of the three cases requesting PGD for CF. White symbols are unaffected individuals and black symbols are affected individuals, N stands for the absence of mutation at a given locus. Numbering of the alleles IVS8CA and IVS17bCA are attributed arbitrarily in each family.

Table 3 Preimplantation genetic results

Embryo	*Embryo grade at day 3*	*No. of cells take*	*F508 locus results*	*IVS8CA results*	*IVS17bCA results*	*Diagnosis*	*Embryo grade at day 4*	*Transfert*	*Confirmatory results (No. cells)*
Case 1									
1	8	2	ΔF508/F508	2/2	1/1	Affected	6	No	Affected (3)
			ΔF508/F508	2/2	1/1				
2	9	2	ΔF508/F508	2/2 or 1/2	1/1	Unclear	5	No	Unclear (2) (b)
			F508/F508(a)	–	–				
3	8	2	ΔF508/F508	–	1/1	Affected	4	No	Affected (2)
			ΔF508/F508	2/2	1/1				
4	8	2	ΔF508/F508	2/2	1/1	Affected	Compacted	No	Affected (3)
			ΔF508/F508	2/2	1/1				
Case 2									
1	8	2	F508/F508	1/1	2/1	Unaffected	9	Yes	–
			F508/F508	1/1	2/1				
2	6	1	F508/ΔF508	2/2	2/1	Affected	6	No	Affected (2)
4	8	2	F508/ΔF508	2/2	2/1	Affected	11	No	Affected (4)
			F508/ΔF508	2/2	2/1				
5	6	2	F508/F508	2/1	2/1	Carrier	6	No	–
			F508/F508	2/1	2/1				
6	8	2	–	–	–	Carrier	8	No	–
			F508/F508	2/1	2/1				
9	8	2	F508/ΔF508	2/2	2/1	Affected	12	No	Affected (3)
			F508/ΔF508	2/2	2/1				
10	8	3 (c)	F508/ΔF508	2/2	2/1	Affected	>20 compacted	No	Affected (embryo)
15	7	1	F508/ΔF508	2/2	2/1	Affected	9	No	Affected (3)
16	8	2	F508/ΔF508	1/2	2/1	Carrier	>10 compacted	Yes	–
			F508/ΔF508 (a)	–	–				
17	7	2	–	–	–	–	5	No	Affected (2)
18	8	2	F508/ΔF508	2/2 or 1/2	2/1	Unclear	5	No	Unclear (4)
			F508/F508 (d)	2/2	1/1				
Case 3									
1	7	2	F508/F508	1/1	1/2	Unaffected	*	Yes	
			F508/F508	1/1	1/2				
2	7	2	ΔF508/F508	1/2	2/2	Affected	10	No	Affected (3)
			ΔF508/F508	1/2	2/2				

(a) Amplification failure in one blastomere tested; (b) No visible nucleus in one cell; (c) Two cells lysed during biopsy; (d) Cell lysed during biopsy transferred at day 3.

we obtained an ambiguous result because of a discordant result between the two blastomeres analysed.

Two embryos were transferred but no pregnancy ensued. Reanalysis of the affected embryos confirmed the initial results, for embryo 18, the genotype remained ambiguous (Table 3).

Case 3: six cumulus-oocyte complexes were retrieved and inseminated by ICSI, all of them exhibited two pronuclei. Three days later two embryos were suitable for biopsy and two blastomeres were successfully removed and analysed. A full diagnosis was obtained on both cells for both embryos resulting in the transfer of one unaffected embryo free of any mutation, no pregnancy ensued. The re-analysis of three blastomeres of the affected embryo confirmed our previous diagnosis (Table 3).

In total, we have analysed 31 blastomeres for which we obtained a PCR signal for 28 (90%). From these 26 (93%), 25 (89%) and 25 (89%) were correctly diagnosed at the loci F508, IVS8CA and IVS17bCA respectively. A full genotype was possible in 22 cases (79%). A complete amplification failure from three blastomeres (10%), a partial amplification failure from two blastomeres (7%) and two ambiguous results (7%) were observed. If we combine these results with the data obtained from the re-analysis, we had 58 out of 62 (94%) blastomeres that presented a PCR signal from which 55 out of 58 (95%), 54 out of 58 (93%) and 55 out of 58 (95%) showed a signal at the F508, IVS8CA and IVS17bCA loci, respectively. A complete haplotype was obtained in 49 out of 58 (84%). A complete amplification failure was observed four times out of 62 (6%). The ADO rate is difficult to estimate because of the two ambiguous results obtained which can be attributed either to ADO or to a chaotic constitution of the embryos (as often observed in IVF embryos[29]) and because of the absence of informativity of the markers.

Discussion

Since the development of new PGD tests is highly time consuming (3 to 6 months for the development of a reliable protocol for a new mutation) and considering that most

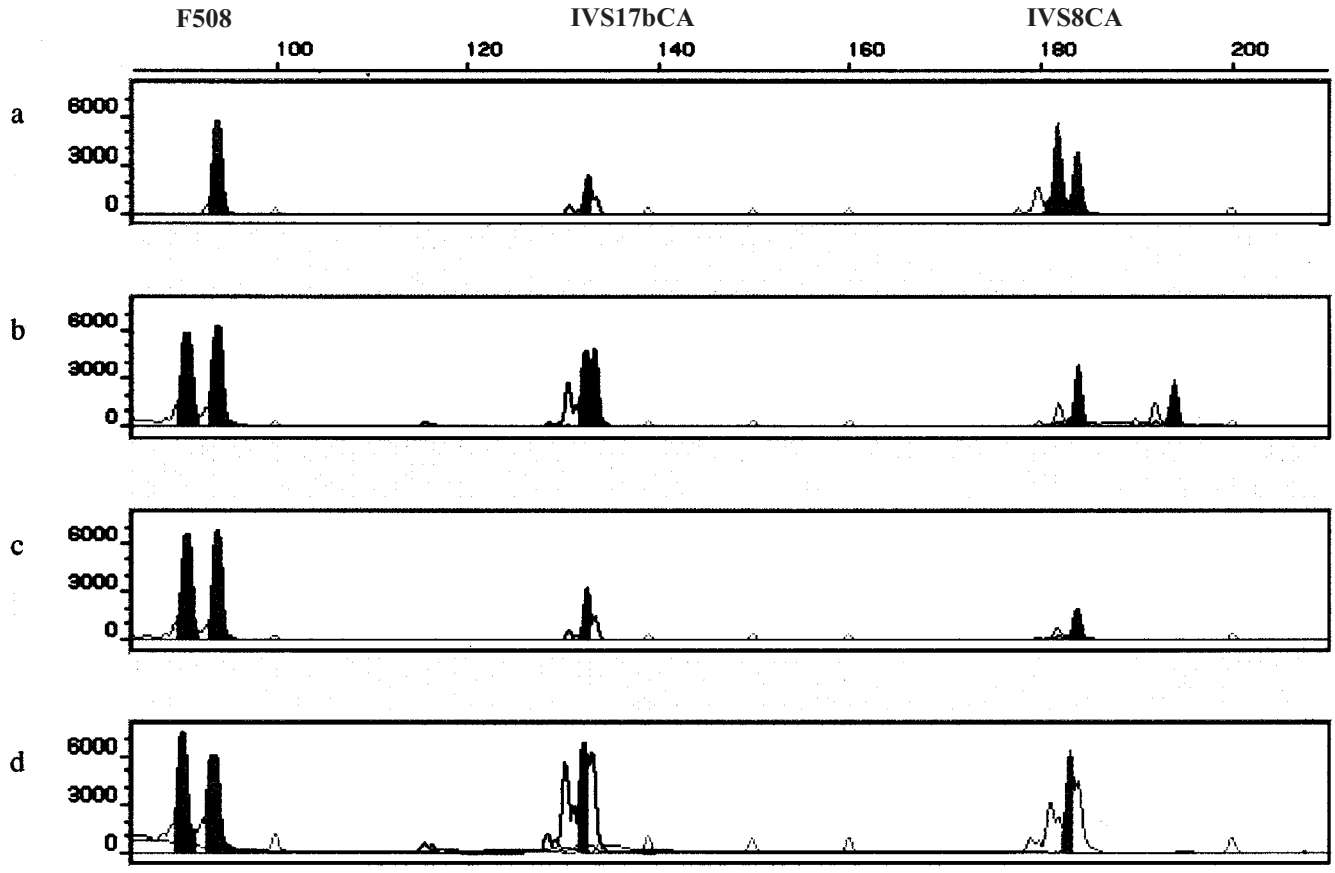

Figure 2 Electrophoregram of multiplex PCR products from case 1. On top are indicated loci's names. Mother's DNA analysis is shown in (**a**), father's DNA analysis in (**b**), affected foetus' DNA in (**c**) and a blastomere from an affected embryo in (**d**).

genetic disorders are due to multiple defects in one gene, it is not reasonable to develop a PGD for each mutation. This is especially true for CF since almost 1000 different mutations have been described. A general solution to this problem is to base the diagnosis on an indirect approach by the characterisation of the morbide allele(s), using linked (intra or extragenic) polymorphic markers, instead of detecting the mutation(s) itself. The advantages of such an approach are: (i) having a single diagnosis procedure for most of the couples requesting PGD whatever mutations are involved, as soon as the couple is informative for the polymorphic markers used; (ii) considerably improving the reliability of the tests since it can give multiple information from a single cell. In the most favourable situation each locus tested can be considered as an independent diagnosis; (iii) possibly detecting amplification problems (ADO or amplification failure) or exogenous DNA contamination; (iv) evaluating the ploidy status of the blastomere tested.

As shown by Lewis *et al*, the misdiagnosis rate is considerably reduced when using a linked maker in addition to the detection of the mutation. This is true for recessive as well as for dominant diseases. The error decreased from 5.8% to 0.44% and from 10.9% to 0.1% respectively when the test is based on the detection of the mutation and a linked marker instead of just the detection of the mutation.[7] The misdiagnosis rate and the reliability of the test will correlate with the number of markers and their informativity. Therefore, the use of linked markers allows PGD for a variety of mutations for the same disorder but also provides safer tests.

The use of polymorphic markers in CF PGD has already been described either in simplex PCR protocols using the markers IVS17bTA or M470V or IVS8CA[20] or in a multiplex PCR protocol using four highly polymorphic makers (D7S523; D7S486; D7S480; D7S490) flanking the CFTR gene[19] or in different duplex combinations involving either markers IVS8CA and IVS17bTA or ΔF508 and IVS17bTA.[21] We describe here a new protocol based on the co-amplification of the ΔF508 and two intragenic polymorphic markers (IVS8CA and IVS17bCA). We decided to coamplify the ΔF508 mutation because, if we consider that its frequency is 70% of the CF mutations, for 91% of the couples requesting PGD for CF at least one of the members of the couple will be a

carrier of the ΔF508 mutation. Furthermore the use of intragenic markers will avoid the problem of recombination since no crossing-over has been reported so far in the CFTR gene. The main advantage of the protocol developed by Dreesen *et al*, resides in the use of highly polymorphic markers.[19] The disavantage is the risk of meiotic recombination since the markers used are located between 0.2 and 5 cM from the CFTR gene. Hence it is necessary to perform an analysis on at least four members, both parents, one affected child and one unaffected sibling and to have at least one informative marker on each side of the CFTR gene for the PGD application.[19] Considering the degree of polymorphism of the markers and the necessity for having at least two informative markers they can, theoretically, propose their protocol to 87% of the couples.[19] The degree of polymorphism of the markers used in this work (48 and 39%) represents the major limitation of the proposed approach. The absence of intragenic meiotic recombination in the CFTR gene considerably simplifies the family analysis compared to the strategy of Dreesen *et al*. However, if we consider that 91% of the couples present at least the ΔF508 mutation, and if we take into account the fact that the ΔF508 locus is co-amplified with the markers IVS8CA and IVS17bCA, our test should be suitable for nearly 80% of the couples requesting PGD for CF. For the couples for which these two markers will not be informative we still have the possibility of offering them a test based on the detection of the ΔF508 mutation only and the elimination of all the embryos carrying this mutation. This represents 42% of the 20% non-informative couples for which only 50% of the embryos will be available for transfer, meaning that a third of the unaffected embryos will be discarded.

Our first experience using this test shows its merits for the PGD of CF since a complete haplotype was obtained in 89% of the blastomeres analysed. Our strategy of coamplifying F508, IVS8CA and IVS17bCA is now used for all our informative couples whatever the mutation is, since it considerably increases the accuracy of the test even if both members of the couple are carriers of the ΔF508 mutation.

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