Molecular Human Reproduction Vol.8, No.9 pp. 880–886, 2002

Multiplex sequence variation detection throughout the CFTR gene appropriate for preimplantation genetic diagnosis in populations with heterogeneity of cystic fibrosis mutations

Christina Vrettou[1,2], Maria Tzetis[1], Joanne Traeger-Synodinos[1], Giles Palmer[1] and Emmanuel Kanavakis[1,3]

[1]Medical Genetics, Athens University and [2]Research Institute for the Study of Genetic and Malignant Disorders in Childhood, St Sophia's Children's Hospital, Athens 11527, Greece

[3]To whom correspondence should be addressed. E-mail ekanavak@cc.uoa.gr

Cystic fibrosis (CF) is one of the most important genetic diseases requiring prevention programmes. Preimplantation genetic diagnosis (PGD) represents an alternative to prenatal diagnosis, and is especially appropriate for couples with an unsuccessful reproductive history. For clinical application, protocols must be optimized to minimize PCR failure, allelic drop-out (ADO) and contamination, while simultaneously detecting a wide spectrum of CF genotypes. We have developed a flexible multiplex PCR protocol allowing analysis of sequence variations in any combination amongst seven CFTR gene exons (4, 10, 11, 13 in two parts, 14b, 17b and 21) by nested PCR and denaturing gradient gel electrophoresis analysis, along with analysis of a fluorescently labelled intragenic microsatellite (IVS8CA). The experiments were carried out on 390 single lymphocytes from three CF patients, one heterozygote and one non-CF individual. PCR efficiency of the exons ranged from 90 to 100%, and ADO from 0 to 3.8%. IVS8CA was co-amplified with a PCR efficiency of 92.4 and 10.8% ADO. The present method overcomes the need for separate assays for each CFTR gene mutation. Additionally, it facilitates analysis of any informative linked polymorphic sequence variation (within the seven exons) along with analysis of a microsatellite, which is useful (when informative) for minimizing misdiagnosis and/or indirect diagnosis. This method proved robust and flexible for diagnosing diverse CF genotype combinations in single cells.

Key words: cystic fibrosis/denaturing gradient gel electrophoresis/mutation analysis/preimplantation genetic diagnosis

Introduction

Cystic fibrosis (CF) is the most common autosomal recessive disease in Caucasians (Welsh *et al*., 1995) and in Greece constitutes the second most common monogenic disorder (after β-thalassaemia and related haemoglobinopathies), with up to 5% of the population being carriers for CF transmembrane conductance regulator (CFTR) gene mutations (authors, unpublished data). It is caused by defects in the CFTR gene (250 kb) located on chromosome 7q31–32 and consisting of 27 exons (Riordan *et al*., 1989; Zielenski *et al*., 1991). The normal CFTR protein comprises 1480 aa and functions as a chloride channel. Defects in the CFTR protein cause an abnormal chloride concentration along the apical membrane of epithelial cells resulting in progressive lung disease, pancreatic dysfunction and male infertility (Welsh *et al*., 1995; Kanavakis *et al*., 1998; Tzetis *et al*., 2000). Infertility in CF males results from congenital bilateral absence of the vas deferens and may even occur in the absence of other clinical CF symptoms. Furthermore, there have been reports of increased frequencies of CF mutations in healthy men with reduced sperm quality and/or obstructive azoospermia, even in the presence of the vas deferens (Anguiano *et al*., 1992; Mercier *et al*., 1995; Van der Ven *et al*., 1996; Meschede and Horst, 1997; Pauer *et al*., 1997; Kanavakis *et al*., 1998).

Prenatal diagnosis and termination of affected pregnancies is the current approach for controlling severe genetic diseases such as CF. An alternative to prenatal diagnosis is the diagnosis and selective transfer of healthy IVF embryos (preimplantation genetic diagnosis, PGD), therefore avoiding the need to terminate ongoing pregnancies. PGD may also be more appropriate for couples with an unsuccessful reproductive history (repeated selected abortions or fertility problems) and especially for those couples who are undergoing assisted reproduction for male infertility and who have the additional risk of transmitting CF.

PGD is usually performed on single blastomere cells biopsied from 6- to 8-cell cleavage stage embryos (Handyside *et al*., 1990; Kanavakis and Traeger-Synodinos, 2002). For PGD of monogenic diseases, diagnostic protocols are based on PCR and have to address the inherent problems of single cell PCR [total PCR failure and allelic drop-out (ADO)], and in addition facilitate the accurate genotyping of the cell for the disease under investigation. In Southern Europe, including Greece, CF is characterized by great allelic heterogeneity compared with the populations of Northern Europe (Kerem *et al*., 1989; Chevalier-Porst *et al*., 1994; Chillon *et al*., 1994; Tzetis *et al*., 1997, 2000; Kanavakis *et al*., 1998). Most of the approaches for PGD

Table I. PCR efficiency and allele drop-out (ADO) in 290 single lymphocyte samples from three CF patients and one heterozygote

Exon	Mutation/ polymorphism	IVS8CA repeats (length bp)	Exons amplified	No. cells product (%)	PCR cells (%)	ADO/total
Patient 1	F508del	25 (196)	10	50	47 (94.0)	0/47 (0)
	621 + 1G→T	23 (192)	4		48 (96.0)	1/48 (2.1)
Patient 2	N1303K	25 (196)	21	85	80 (94.1)	3/80 (3.8)
	2789 + 5G→A	18 (182)	14b		85 (100)	2/85 (2.4)
Patient 3	E822X	17 (180)	13 part b	80	72 (90.0)	1/72 (1.4)
	F1052V	18 (182)	17b		75 (93.8)	2/75 (2.6)
Heterozygote[a]	1719–9T→C	17 (180)	11	75	75 (100.0)	0/75 (0)
	R668C		13 part a			
	Normal allele	18 (182)			74 (98.7)	1/74 (1.4)
Microsatellite IVS8CA				290	268 (92.4)	29/268 (10.8)

[a]Individual heterozygote for D565G mutation in exon 12 (not included in assay) had two polymorphisms *in cis* to D565G (1719–9T→C in exon 11 and R668C in exon 13 part a), which were also *in cis* with 17 CA repeats in IVS8. The normal allele was *in cis* to 18 CA repeats in IVS8.

of CF that have been described to date have either not addressed the problem of diagnosing a wide spectrum of mutation combinations (Liu *et al*., 1993; Ao *et al*., 1996; Schaaff *et al*., 1996; Scobie *et al*., 1996; Tsai *et al*., 1999) or require the design of case-specific protocols each time (Goossens *et al*., 2000). Protocols based on the analysis of CFTR gene-linked polymorphic markers have been described which are quite widely applicable for some Northern European populations (Dreesen *et al*., 2000; Eftedal *et al*., 2001), although their usefulness has not been assessed elsewhere.

In this study, our aim was to develop a practical, flexible PGD protocol appropriate for analysis of a wide spectrum of potentially affected genotypes in the Greek population, precluding the design of case-specific protocols each time. Exons 4, 10, 11, 13 (in two parts, a and b), 14b, 17b and 21 of the CFTR gene contain ~47 different mutations accounting for 81% of CF chromosomes in Greece (Tzetis *et al*., 1997; authors, unpublished data). All mutations within these exons can be detected with denaturing gradient gel electrophoresis (DGGE) analysis (Fanen *et al*., 1992), which as a scanning method can detect any sequence variation in a single amplified region. For that reason, we standardized a protocol involving first round multiplex PCR (for any combination of the seven CFTR exons) followed by nested PCR/s producing amplicons analysable by DGGE. Polymorphic sequence variation(s) present in any of the seven exons can also be detected by DGGE and exploited, through linkage, for indirect analysis and/or to cross-check the genotype. A fluorescently labelled intragenic polymorphic microsatellite, IVS8CA (71% expected heterozygosity in the Greek population; authors, unpublished data), was included in the multiplex PCR to facilitate confirmation of the genotype or for indirect diagnosis (when informative).

Materials and methods

Cell samples

Experiments were carried out on a total of 390 single lymphocytes: 290 lymphocytes from three CF patients and one CF heterozygote (carrier for one CF mutation and two polymorphisms) (Table I) and 100 lymphocytes from a non-affected individual. The lymphocytes were isolated from the nucleated cell phase, after density gradient centrifugation at 800 *g* of 2.5 ml of whole blood diluted with an equal volume of 0.9% NaCl, using Ficoll separating solution (Density 1.077, Seromed; Biochrom KG, Berlin, Germany) (Vrettou *et al*., 1999).

Manipulation of all cell types was performed under a binocular dissecting microscope (Nikon SMZ-U) using finely pulled glass pipettes. Each cell sample was rinsed six times in microdrops of IVF medium (Scandanavian IVF Science, AB, Sweden) and placed in 10 µl of double-distilled water in DNAse-free, RNAse-free 0.2 ml Eppendorf tubes prior to further processing (Vrettou *et al*., 1999).

Following cell selection, samples were frozen at –20°C until time of cell lysis and PCR set-up.

Cell lysis

A total of 5 µl of PCR-grade Proteinase K (Roche Molecular Biochemicals, Germany), diluted in double-distilled water, was added to the 10 µl of water containing the selected cell, to a final concentration of 50 µg/ml. The enzyme was activated by incubation at 37°C for 1 h followed by 65°C for 10 min, and inactivated by heating to 95°C for 10 min (Traeger-Synodinos *et al*., 1997).

Genotype analysis

The protocol involved a first round multiplex PCR (2.5 h), containing PCR primers for the appropriate exons (selected according to the genotype under investigation) as well as for the IVS8CA microsatellite, followed by (i) nested PCR to produce DGGE analysable fragment(s) (2.5 h) and electrophoresis on a denaturing gradient gel (5 h), and in parallel (ii) size analysis of the fluorescently labelled microsatellite marker (3 h).

Initial experiments were set up to investigate the efficiency of exon amplification (but not ADO) in multiplex PCR reactions for various CFTR exons in the following combinations: (i) exons 4, 10, 11 and 21 (containing 72.1% of the mutations in the Greek population) and (ii) exons 13 (in two parts, a and b), 14b and 17b (containing an additional 8.5%). PCR primers for the microsatellite marker IVS8CA were included with each combination. The experiments were performed on single lymphocytes from a non-CF individual using identical PCR conditions for both primer sets. Separate nested PCR reactions for each exon followed the multiplex PCR, and successful amplification was indicated by the presence of a correctly sized PCR product on agarose gel electrophoresis. Efficiency of co-amplification of the IVS8CA microsatellite in the first-round multiplex PCR was evaluated by detection of an appropriately sized PCR product on an automatic DNA sequencer.

Once satisfactory amplification of all exons and the IVS8CA microsatellite was established, the PCR efficiency of other (any) exon combinations and the rate of ADO were evaluated by applying the method to genotype single lymphocytes from three CF patients and one CF heterozygote, with known mutations/polymorphisms in the CFTR gene (Table I). DGGE analysis of the appropriate exons simultaneously confirmed the accuracy of genotype analysis and ADO rates for exons. In addition, the lymphocyte donors (excluding the non-CF individual) were selected to have two different allele sizes for the IVS8CA microsatellite marker, for evaluation of ADO for this site.

First-round multiplex PCR

PCR primers for the first round multiplex PCR for the seven CFTR exons and for the fluorescent amplification of the IVS8CA microsatellite were as previously described (Zielenski *et al*., 1991; Morral and Estivill, 1992) (Table II).

Table II. Primer list

First round multiplex PCR primers

CFTR exons	Primers size (bp)	Sequence of primers	Product
4	f 4i-5	5′-TCACATATGGTATGACCCTC-3′	438
	r 4i-3	5′-TTGTACCAGCTCACTACCTA-3′	
10	f 10i-5	5′-GCAGAGTACCTGAAACAGGA-3′	491
	r 10i-3	5′-CATTCACAGTAGCTTACCCA-3′	
11	f 11i-5	5′-CAACTGTGGTTAAAGCAATAGTGT -3′	425
	r 11i-3	5′-GCACAGATTCTGAGTAACCATAAT-3′	
13 part a	f 13i-5	5′-TGCTAAAATACGAGACATATTGCA-3′	528
	rC1–1M	5′-ATCTGGTACTAAGGACAG-3′	
13 part b	fX13B-5	5′-TCAATCCAATCAACTCTATACGAA-3′	497
	r 13i-3A	5′-TACACCTTATCCTAATCCTATGAT-3′	
14b	f 14Bi-5	5′-GAACACCTAGTACAGCTGCT-3′	449
	r14Bi-3	5′-AACTCCTGGGCTCAAGTGAT-3′	
17b	f 17bi-5	5′-TTCAAAGAATGGCACCAGTGT-3′	463
	r 17b-3	5′-ATAACCTATAGAATGCAGCA-3′	
21	f 21i-5	5′-AATGTTCACAAGGGACTCCA-3′	477
	r 21i-3	5′-CAAAAGTACCTGTTGCTCCA-3′	
Microsatellite marker			
IVS8CA	AC8R4	5′-ACTAAGATATTTGCCCATTATCAAGT-3′	170–198
	AC8D3	Texas Red 5’-TCTATCTCATGTTAATGCTG-3′	

Nested PCR (DGGE) primers

CFTR exons	Primers temp. (°C)	Sequence of primers size (bp)	Annealing	Product
4	f CF4	5′-TGTGTTGAAATTCTCAGGGT-3′	55	369
	r GCCF4	5′-(40GC)CAGAATATATGTGCCATGGG-3′		
10	f CF10	5′-TCCTGAGCGTGATTTGATAA-3′	55	336
	r GCCF10	5′-(35GC)ATTTGGGTAGTGTGAAGGG-3′		
11	f GCCF11	5′-(35GC)CAGATTGAGCATACTAAAAGTG-3′	50	224
	r CF11	5′-CATTTACAGCAAATGCTTGCTAG-3′		
13 part a	f GCCF1311	5′-(35GC)TATATCTTAAAGCTGTGTCTGT-3′	55	516
	r CF1312	5′-TCCCTGCTCAGAATCTGGTA-3′		
13 part b	f GCCF1321	5′-(50GC)CCCTTACAAATGAATGGCAT-3′	50	454
	r CF1323	5′-TACATATTGCATTCTACTCA-3′		
14b	f CF14B	5′-AATAGGTGAAGATGTTAGAA-3′	50	168
	r GCCF14B	5′-(40GC)ATAAAACACAATCTACACAA-3′		
17b	f GCCF17B	5′-(40GC)TTTGTGTTTATGTTATTTGC-3′	50	266
	r CF17Bτ	5′-ATCATTTCTATTCTCATTTG-3′		
21	f CF21	5′-TGAAATATTTTACAATACAATAAGGG-3′	55	272
	r GCCF21	5′-(40GC)GCCATTTGTGTTGGTATGAG-3′		

A long initial denaturation time was applied to ensure complete denaturation of genomic template DNA (Ray and Handyside, 1996), facilitated by the use of the modified heat-activated form of a recombinant Taq DNA polymerase (HotStarTaq; Qiagen) and followed by a protocol of short PCR cycling times based on a previously published method (Vrettou *et al*., 1999). PCR reactions were carried out in 50 µl volumes containing 30 µl of the premixed solution of buffer/dNTPs/$MgCl_2$/Taq polymerase provided by the manufacturer and 1 µmol/l of each primer, except for the fluorescent primer which was added to a concentration of 0.3 µmol/l. The PCR cycles were: initial denaturation of 15 min at 95°C, followed by 20 cycles of 96°C for 30 s, 55°C for 40 s and 72°C for 30 s and 20 more cycles of 96°C for 30 s, 55°C for 20 s and 72°C for 30 s.

Between 1 and 2 µl of the fluorescent (Texas Red) tagged PCR-generated template of polymorphic dinucleotide microsatellite marker (IVS8CA) (Morral and Estivill, 1992) was then electrophoresed in a 7% denaturing acrylamide gel (32% formamide, 5.6 mol/l urea, in TBE) and was sized (and analysed) on a Vistra automatic DNA sequencer 725 using the Fragmentor software (Molecular Dynamics/Amersham Life Sciences, UK).

Nested PCR and DGGE analysis

Nested PCR primers for DGGE analysis of exons were as previously described (Fanen *et al*., 1992) (Table II). In all experiments exons were separately reamplified (according to the genotype of the sample under investigation) using 1 µl of the first round of PCR in a 50 µl volume containing a premixed solution of buffer/dNTPs/$MgCl_2$/Taq polymerase provided by the manufacturer (HotStarTaq; Qiagen) and 0.4 µmol/l of each primer appropriate for DGGE analysis (Fanen *et al*., 1992). The PCR cycling conditions were 95°C for 15 min followed by 40 cycles of 30 s at 95°C, 20 s at the appropriate annealing temperature for each exon (Table II) and 30 s at 72°C. Following the nested PCR, 10 µl of amplification products were checked by agarose gel electrophoresis and mutations/polymorphisms were analysed by DGGE as previously described (Fanen *et al*., 1992).

Precautions against contamination

In all experiments, precautions against contamination were stringent. Manipulation of cells and PCR set-up were carried out in separate UV-treated laminar flow hoods. PCR set-up employed exclusive PCR pipettes and pipette tips with filters. A maximum of 10 single cell samples was analysed per experiment. In the first round PCR, one tube containing IVF medium and cell lysis mixture only and one tube containing PCR mixture only were included as negative (blank) controls. In the nested PCR set-up (which included nesting the two negative controls from the first PCR), an additional two blanks containing PCR mixture only were included for all experiment sets.

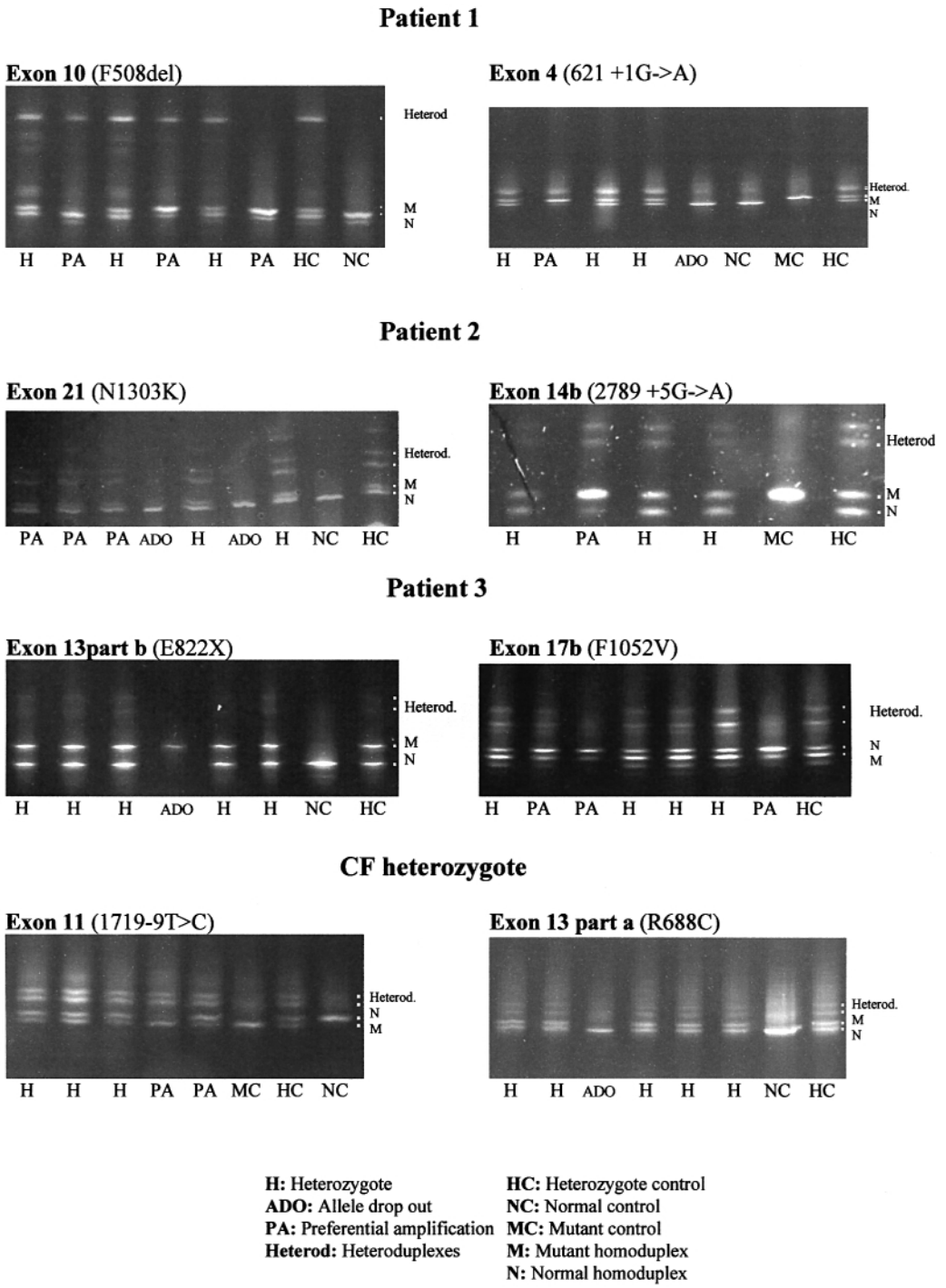


Figure 1. DGGE analysis of the mutations/polymorphisms of single lymphocytes from the four lymphocyte donors. (Note: DGGE gels for exons 10 and 17b sporadically demonstrated non-specific electrophoretic bands below the homoduplex bands.)

Results

In the two multiplexed CFTR exon sets (50 single lymphocytes from the non-CF individual each), PCR efficiency for each exon was 94% (exon 4), 92% (exon 10), 98% (exon 11), 96% (exon 21), 98% (exon 13, part a), 92% (exon 13, part b), 98% (exon 14b) and 94% (exon 17b). Co-amplification of IVS8CA showed 91% PCR efficiency with exons 4, 10, 11 and 21, and 94% PCR efficiency with exons 13 (part a and b), 14b and 17b. This non-CF individual was homozygous for the allele size of IVS8CA.

DNA from single lymphocytes of the three CF patients and the one CF heterozygote was amplified (using nested PCR) for the appropriate (two) exons according to the genotype, and also for the IVS8CA marker (Figures 1 and 2). ADO rates for exons were evaluated following DGGE analysis, and ADO was scored when a single homoduplex band was observed (Figure 1). Occasional samples gave patterns with faint homoduplex and/or heteroduplex bands (Figure 1), implicating preferential amplification from one of the alleles (Findlay *et al*., 1995). This was not considered to represent ADO (Figure 1). The results of PCR efficiency and ADO are summarized in Table I.

PCR products were not observed in any of the negative controls (blanks) amongst the 30 sets of up to 10 cells that were analysed

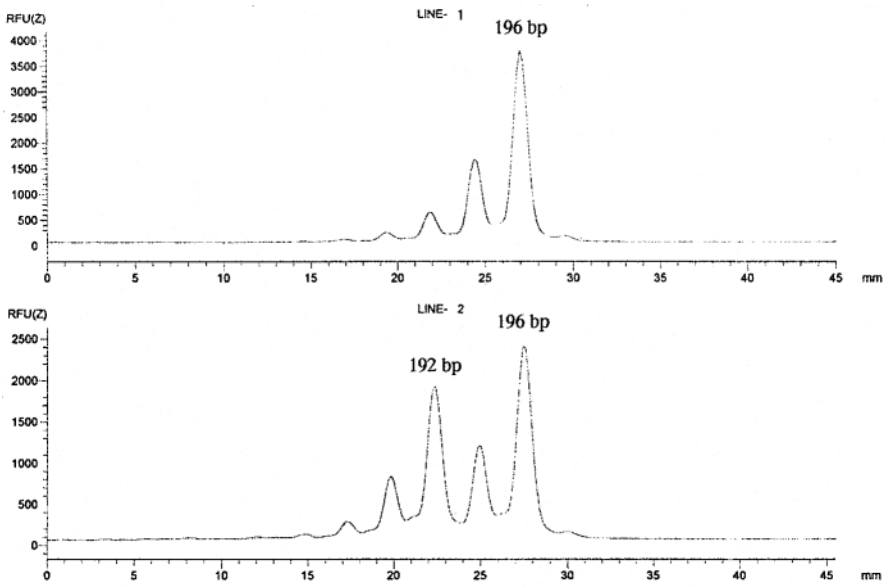


Figure 2. Analysis of fluorescently labelled dinucleotide microsatellite IVS8CA of single lymphocytes from patient 1 (F508del/621+1G→T). Line 2 (bottom): double heterozygous pattern of the 196 and 192 bp alleles which represent 25 and 23 CA repeats respectively; line 1 (top): ADO of the 192 bp allele.

(i.e. 60 blank tubes from the first PCR and 120 blank tubes from the nested PCR reactions), indicating that contamination, from external sources or PCR carry-over, was not a problem with the stringent precautions applied.

Discussion

CF is targeted as one of the priority diseases for prevention programmes, but prenatal diagnosis with termination of affected pregnancies is not always a preferable option, and PGD represents an alternative approach. The development of a PGD protocol for CF is additionally useful because of the association of some forms of male infertility with mutations in the CFTR gene. In such cases, female partners should also be screened for CFTR gene mutations and in cases where they are both carriers, PGD can be considered in combination with the assisted reproduction procedure.

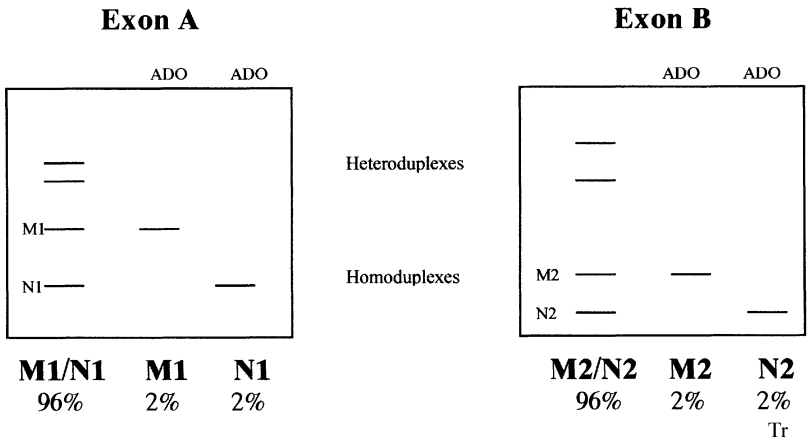
Most of the described approaches for PGD of CF are based on designing protocols for the most frequent CF mutations or case-specific protocols (Liu *et al*., 1993; Ao *et al*., 1996; Schaaff *et al*., 1996; Tsai, 1999; Goossens *et al*., 2000). For a common disorder such as CF and associated atypical forms (such as male infertility) where as many as 40–50 mutations may account for ~80% of CF alleles (especially for the Southern European populations) (Chevalier-Porst *et al*., 1994; Chillon *et al.*, 1994), it is more practical to develop a widely applicable diagnostic protocol than to design case-specific methods each time. A potentially appropriate method that uses indirect genotyping through linkage analysis of extragenic CFTR gene markers has been described (Dreesen *et al*., 2000), but it has only been evaluated for the Dutch and French Canadian populations, and there are no data on its usefulness in other populations. Our study evaluates an alternative strategy for simultaneously detecting sequence variations in up to seven different exons in the CFTR gene, along with size analysis of a linked microsatellite marker.

PCR efficiency for all exons and the microsatellite marker (IVS8CA) was >90% in all six different multiplex PCR reactions (two sets of four exons then four sets of two exons, see Table I). ADO amongst the seven CFTR exons ranged from 0 to 3.8%, all well within acceptable limits for single-cell PCR (ESHRE PGD Consortium Steering Committee, 2001). Paradoxically, ADO for the fluorescent PCR of IVS8CA was much higher (10.8%) than for the conventional PCR of the exons, which is contrary to the expected advantages of using fluorescent PCR. A possible reason for this is that the annealing temperature used in the multiplex reaction was optimal for the exons, but higher than optimal (by 5°C) for the microsatellite marker primers (Morral and Estivill, 1992). Despite this, the ADO for IVS8CA is still within the acceptable range for PGD application.

For clinical PGD cycles when parental genotypes involve two different CFTR mutations (or the same mutation) within the same exon, DGGE facilitates the detection of both alleles simultaneously such that the presence of a normal band (as part of a heterozygote pattern or alone) insures a non-affected embryo for transfer, even if ADO has occurred (Vrettou *et al*., 1999). For genotypes including two mutations in different exons, the presence of a mutant band in both fragments (as part of a heterozygote pattern, or alone if ADO has occurred) indicates an affected genotype (Figure 3). Although the absence of a mutant band in one or both of the two exons indicates an unaffected genotype (heterozygous or normal genotype respectively), there may be a risk of an unacceptable misdiagnosis due to ADO.

The ADO observed in this study (between 0 and 3.8% in exon 21, Table I) was very low in comparison with other reports (Findlay *et al*., 1995; Goossens *et al*., 2000), although it remains a possible cause of misdiagnosis including the danger of rejecting a healthy embryo or transferring an affected embryo. The theoretical possibility of misdiagnosis was calculated, taking into consideration an over-estimated maximum ADO rate of ~4% for all fragments. When the two mutations are in the same exon, failure to amplify the normal allele may lead to rejection of 1% of heterozygous embryos (with no unacceptable consequences) (Vrettou *et al*., 1999). In cases where the two mutations are in different exons, the amplification of independent regions of the gene are each subject to the random occurrence of ADO and this may lead to a 0.0396 possibility of transferring an affected embryo for implantation (Figure 3). This was calculated as the sum of the products of the possibilities of 'unaffected' combinations of DGGE patterns being in fact 'affected' (Figure 3). The above possibility refers only to misdiagnosing affected genotypes/embryos,

Possible combination of DGGE patterns of affected embryos when mutations occur in different exons



Combinations		Diagnosis	Possibility	
			Correct diagnosis (Rejection of affected embryo)	**Misdiagnosis** (Transfer of affected embryo)
M1/N1	M2/N2	A (R)	0.9216	
M1/N1	M2	A (R)	0.0192	
M1/N1	N2	N (T)		0.0192
M1	M2/N2	A (R)	0.0192	
M1	M2	A (R)	0.0004	
M1	N2	N (T)		0.0004
N1	M2/N2	N (T)		0.0192
N1	M2	N (T)		0.0004
N1	N2	N (T)		0.0004
		Total	0.9604	0.0396

M1: Mutation in exon A
M2: Mutation in exon B
A: Diagnose as affected embryo
N: Diagnose as non affected embryo
N1: Wild type of exon A
N2: Wild type of exon B
R: Combination of patterns leading to reject an affected embryo
T: Combination of patterns leading to transfer an affected embryo

Figure 3. Theoretical calculation of the possibility to misdiagnose and transfer an affected embryo, combined heterozygote for the mutations M1 and M2, which occur in different exons A and B respectively. Calculations refer to the potentially affected embryos (25% of all the embryos to be analysed) and is based on the chance of observing each combination of DGGE analysis patterns, due to ADO, as illustrated in the figure. For this calculating approach, the maximum ADO rate of 4% which was observed in trials (exon 21) was used for both exons even though most exons had negligible ADO (Table I). The total risk of misdiagnosing an affected embryo is 0.0396 which reflects 0.99% (<1%) of all embryos tested.

which theoretically account for 25%; thus for all embryos tested, the overall possibility of misdiagnosis is <1%.

DGGE analysis is an accurate, reliable method with relatively low running costs, and is amongst the methods commonly chosen for routine genotyping and prenatal diagnosis for many monogenic disorders (Losekoot *et al*., 1990; Fanen *et al*., 1992; Kanavakis *et al*., 1997; Nissen *et al*., 1998; Romstad *et al*., 2000). In addition, our laboratory has been successfully applying an analogous DGGE-based genotyping strategy for PGD of β-thalassaemia for the last 3 years (Kanavakis *et al*., 1999). The calculated risk of misdiagnosis with unacceptable consequences due to ADO for this method (in those cases when the two mutations are in different exons) was based on an overestimated ADO rate of 4%, implying that, in practice, the risk of misdiagnosis with this method is much less than 1%. Additionally, the analysis of the linked polymorphic sequence variations (when informative) should further minimize the chance of misdiagnosis in PGD cycles. Thus this method represents an extremely robust and flexible approach for the application of PGD for diverse genotype combinations; this has not been so far addressed for Southern European populations by any previously published method.

Acknowledgements

The authors wish to thank Konstantina Pouliou and Stavroula Papadopoulou for technical assistance. This work was partly funded by an Athens University Research Grant (ELKE no. 70/4/4255). C.V. is supported by a post-doctoral scholarship from the Greek State Scholarship Foundation.

References

Anguiano, A., Oates, R.D., Amos, J.A., Dean, M., Gerrard, B., Stewart, C., Maher, T.A., White, M.B. and Milunsky, A. (1992) Congenital bilateral absence of the vas deferens: A primarily genital form of cystic fibrosis. *J. Am. Med. Assoc.*, **267**, 1794–1797.

Ao, A., Ray, P., Harper, J., Lesko, J., Paraschos, T., Atkinson, G., Soussis, I., Taylor, D., Handyside, A., Hughes, M. *et al*. (1996) Clinical experience with preimplantation genetic diagnosis of cystic fibrosis (ΔF508). *Prenat. Diagn*., **16**, 137–142.

Chevalier-Porst, F., Bonardot, A.M., Gilly, R., Chazalette, J.P., Mathieu, M. and Bozon, D. (1994) Mutation analysis in 600 French cystic fibrosis patients. *J. Med. Genet.*, **31**, 541–544.

Chillon, M., Casals, T., Gimenez, J., Ramos, M.D., Palacio, A., Morral, N., Estivill, X. and Nunes, V. (1994) Analysis of the CFTR gene confirms the high genetic heterogeneity of the Spanish population: 43 mutations account for only 78% of CF chromosomes. *Hum. Genet.*, **93**, 447–451.

Dreesen, J.C.F.M., Jacobs, L.J.A.M., Bras, M., Herbergs, J., Dumoulin, J.C.M., Geraedts, J.P.M., Evers, J.L.H. and Smeets, H.J.M. (2000) Multiplex PCR of polymorphic markers flanking the CFTR gene; a general approach for pre-implantation genetic diagnosis of cystic fibrosis. *Mol. Hum. Reprod.*, **6**, 391–396.

Eftedal, I., Schwartz, M., Bendtsen, H., Andersen, A.N. and Ziebe, S. (2001) Single intragenic microsatellite preimplantation genetic diagnosis for cystic fibrosis provides positive allele identification of all CFTR genotypes for informative couples. *Mol. Hum. Reprod.*, **7**, 307–312.

ESHRE PGD Consortium Steering Committee (2001) Data collection II. *Hum. Reprod.*, **17**, 233–246.

Fanen, P., Ghanem, N., Vidaud, M., Besmond, C., Martin, J., Costes, B., Plassa, F. and Goossens, M. (1992) Molecular characterization of cystic fibrosis: 16 novel mutations identified by analysis of the whole cystic fibrosis conductance transmembrane regulator (CRTR) coding regions and splice site junctions. *Genomics*, **13**, 770–776.

Findlay, I., Ray, P., Quirke, P., Rutherford, A. and Richard, L. (1995) Allelic drop-out and preferential amplification in single cells and human blastomeres: implications for preimplantation diagnosis of sex and cystic fibrosis. *Mol. Hum. Reprod.*, **1**, 1609–1618.

Goossens, V., Sermon, K., Lissens, W., Vandervorst, M., Vanderfaeillie, A., De Rijcke, M., De Vos, A., Henderix, P., Van de Velde, H., Van Steirteghem, A. *et al*. (2000) Clinical application of preimplantation genetic diagnosis for cystic fibrosis. *Prenat. Diagn*., **20**, 571–581.

Handyside, A.H., Kontogianni, E.H., Hardy, K. and Winston, R.M. (1990) Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. *Nature*, **344**, 768–770.

Kanavakis, E. and Traeger-Synodinos, J. (2002) Preimplantation genetic diagnosis in clinical practice. *J. Med. Genet.*, **39**, 6–11.

Kanavakis, E., Traeger-Synodinos, J., Vrettou, C., Maragoudaki, E., Tzetis, M. and Kattamis, C. (1997) Prenatal diagnosis of the thalassemia syndromes by rapid DNA analytical methods. *Mol. Hum. Reprod*., **3**, 523–528.

Kanavakis, E., Tzetis, M., Antoniadi, Th., Pistofisis, G., Milligos, S. and Kattamis, C. (1998) Cystic fibrosis mutation screening in CBAVD patients and men with obstructive azoospermia or severe oligospermia. *Mol. Hum. Reprod.*, **4**, 333–337.

Kanavakis, E., Vrettou, C., Palmer, G., Tzetis, M., Mastrominas, M. and Traeger-Synodinos, J. (1999) Preimplantation genetic diagnosis in 10 couples at risk for transmitting β-thalassemia major: clinical experience including the initiation of six singleton pregnancies. *Prenat. Diagn.*, **19**, 1217–1222.

Kerem, E., Rommens, J., Buchanan, J.A., Markiewicz, D., Cox, T.K., Chakravarti, A., Buchwald, M. and Tsui, L.C. (1989) Identification of the cystic fibrosis gene: genetic analysis. *Science*, **245**, 1073–1080.

Liu, J., Lissens, W., Devroey, P., Van Steirteghem, A. and Liebaers, I. (1993) Polymerase chain reaction analysis of the cystic fibrosis ΔF508 mutation in human blastomeres following oocyte injection of a single sperm from a carrier. *Prenat. Diagn*., **13**, 873–880.

Losekoot, M., Fodde, R., Harteveld, C.L., van Heeren, H., Giordano, P.C. and Bernini, L.F. (1990) Denaturing gradient gel electrophoresis and direct sequencing of PCR amplified genomic DNA: a rapid and reliable diagnostic approach to beta thalassemia. *Br. J. Haematol.*, **76**, 269–274.

Mercier, B., Verlingue, C., Lissens, W., Silber, S.J., Novelli, G., Bonduelle, M., Audrezet, M.P. and Ferec, C. (1995) Is congenital bilateral absence of vas deferens a primary form of cystic fibrosis? Analyses of the CFTR gene in 67 patients. *Am. J. Hum. Genet.*, **56**, 272–277.

Meschede, D. and Horst, J. (1997) The molecular genetics of male infertility. *Mol. Hum. Reprod.*, **3**, 419–430.

Morral, N. and Estivill, X. (1992) Multiplex PCR amplification of three microsatellites within the CFTR gene. *Genomics*, **13**, 1362–1364.

Nissen, H., Hansen, A.B., Guldberg, P., Hansen, T.S., Petersen, N.E. and Horder, M. (1998) Evaluation of a clinically applicable mutation screening technique for genetic diagnosis of familial hypercholesterolemia and familial defective apolipoprotein B. *Clin. Genet.*, **53**, 433–439.

Pauer, H.U., Hinney, B., Michelmann, H.W., Krasemann, E.W., Zoll, B. and Engel, W. (1997) Relevance of genetic counselling in couples prior to intracytoplasmic sperm injection. *Hum. Reprod.*, **12**, 1909–1912.

Ray, P.F. and Handyside, A.H. (1996) Increasing the denaturation temperature during the first cycles of amplification reduces allele drop out from single cells for preimplantation diagnosis. *Mol. Hum. Reprod.*, **2**, 213–218.

Riordan, J.R., Rommens, J.M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N. and Chou, J.L. (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science*, **245**, 1066–1073.

Romstad, A., Kalkanoglu, H.S., Coskun, T., Demirkol, M., Tokatli, A., Dursun, A., Baykal, T., Ozalp, I., Guldberg, P. and Guttler, F. (2000) Molecular analysis of 16 Turkish families with DHPR deficiency using denaturing gradient gel electrophoresis (DGGE). *Hum. Genet.*, **107**, 546–553.

Schaaff, F., Wedemann, H. and Schwinger, E. (1996) Analysis of sex and DF508 in single amniocytes using primer extension preamplification. *Hum. Genet.*, **98**, 158–161.

Scobie, G., Woodroffe, B., Fishel, S. and Kalsheker, N. (1996) Identification of the five most common cystic fibrosis mutations in single cells using a rapid and specific differential amplification system. *Mol. Hum. Reprod.*, **2**, 203–207.

Traeger-Synodinos, J., Palmer, G., Tzetis, M., Mastrominas, M., Kanavakis, E. and Katamis, C. (1997) Preliminary studies on the feasibility of preimplantation diagnosis for beta-thalassemia. *Medizinische Genetic*, 9 (Suppl) 60.

Tsai, Y.H. (1999) Cost-effective one-step PCR amplification of cystic fibrosis DF508 fragment in a Single cell for preimplantation genetic diagnosis. *Prenat. Diagn*., **19**, 1048–1051.

Tzetis, M., Kanavakis, E., Antoniadi, Th., Doudounakis, S., Adam, G. and Kattamis, C. (1997) Characterization of more than 85% of cystic fibrosis alleles in the Greek population, including five novel mutations. *Hum. Genet.*, **99**, 121–125.

Tzetis, M., Efthymiadou, A., Strofalis, S., Psychou, P., Dimakou, A., Pouliou, E., Doudounakis, S. and Kanavakis, E. (2000) CFTR gene mutations—including three novel nucleotide substitutions—and haplotype background in patients with asthma, disseminated bronchiectasies and chronic obstructive pulmonary disease. *Hum. Genet.*, **108**, 216–221.

Tzetis, M., Efthymiadou, A., Doudounakis, S. and Kanavakis, E. (2001) Qualitative and quantitative analysis of mRNA associated with four putative splicing mutations (621+3A>G, 2751+2T>A, 296+1G>C, 1717–9T> C-D565G) and one nonsense mutation (E822X) in the CFTR gene. *Hum. Genet.*, **109**, 592–601.

Van der Ven, K., Messer, L., Van der Ven, H., Jeyendran, R.S. and Ober, C. (1996) Cystic fibrosis mutation screening in healthy men with reduced sperm quality. *Hum. Reprod.*, **11**, 513–517.

Vrettou, C., Palmer, G., Kanavakis, E., Tzetis, M., Antoniadi, Th., Mastrominas, M. and Traeger-Synodinos, J. (1999) A widely applicable strategy for single cell genotyping of β-thalassaemia mutations using DGGE analysis: application to preimplantation genetic diagnosis. *Prenat. Diagn*., **19**, 1209–1216.

Welsh, M.J., Tsui, L-C., Boat, T.F. and Beaudet, A.L. (1995) Cystic Fibrosis. In Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (eds) *The Metabolic and Molecular Bases of Inherited Disease*. 7th edn, McGraw-Hill, New York, USA, pp. 3799–3876.

Zielenski, J., Rozmahel, R., Bozon, D., Kerem, B-S., Grzelczak, Z., Riordan, J.R., Rommens, J. and Tsui, L.-C. (1991) Genomic DNA sequence of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. *Genomics*, **10**, 214–222.

Submitted on August 28, 2001; resubmitted on February 13, 2002; accepted on May 22, 2002