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Multiple mutation analysis of the cystic fibrosis gene in single cells

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PGD is becoming an alternative to prenatal diagnosis. The combination of IVF techniques with the PCR technology allows for the detection of genetic abnormalities in first polar bodies from oocytes and blastomeres from cleavage-stage embryos. Dealing with a genetic disease with a heterogeneous spectrum of mutations like cystic fibrosis, one of the objectives of centres offering PGD is the application of simple and efficient protocols that allow for the detection of a wide range of mutations with a single procedure. In the present work, 29 normal loci and the 31 most frequent cystic fibrosis transmembrane conductance regulator (*CFTR*) mutations in Southern Europe could be detected at the same time in single cells applying a modified and improved primer extension preamplification-PCR. Two different *Taq* polymerases were tested in isolated buccal cells heterozygous for several mutations. The protocol that gave statistically significant better results was also successful in oocytes and their first polar bodies.

Key words: cystic fibrosis/embryo/first polar body/PEP-PCR/PGD

Introduction

Cystic fibrosis is the most common autosomal-recessive disease which affects one in every 2500 newborns (Cutting, 1997). Its main features are elevation of chloride in sweat, pulmonary disease and pancreatic failure. The gene responsible, cystic fibrosis transmembrane conductance regulator (*CFTR*), was cloned in 1989 (Riordan *et al.*, 1989). The most frequent mutation in approximately 66% of all cases is a 3 bp deletion ΔF508, but over 1000 different mutations have been described (http://www.genet.sickkids.on.ca/cftr-cgi-bin/FullTable). About 1 in 25–30 Caucasians is a carrier of a cystic fibrosis mutation. PGD is an alternative to prenatal diagnosis for the detection of chromosomal abnormalities or single gene defects and can prevent termination of pregnancy in the case of an affected fetus. PGD has been widely applied in cleavage-stage embryos by analysing one or two of their blastomeres (Sermon *et al.*, 2005). PGD can also be performed by first polar body biopsy (PGD-1PB) (Strom *et al.*, 1990). The 1PB is extruded during the first meiotic division and contains the complementary genotype to the oocyte arrested in metaphase II (MII). Its removal has no effect on the subsequent embryo since it is destined to degenerate and does not interfere with fertilization rates or the percentage of embryos entering cleavage (Verlinsky and Kuliev, 1992; Magli *et al.*, 2004). In a female carrying a genetic disease, the absence or presence of the mutation can be detected indirectly in the oocyte by analysis of the 1PB. Only embryos resulting from genetically normal oocytes are transferred back to the mother. To date, PGD-1PB has been applied to several monogenic diseases (Wells and Sherlock, 1998) and its analysis must contemplate recombination events, which would lead to heterozygous gametes. The phenomenon of allele drop-out (ADO) also has to be considered in single-cell PCR (Findlay *et al.*, 1995). It consists of the amplification of only one allele in a heterozygous single cell which could lead to misdiagnosis. The accuracy of the procedure has been improved by including techniques such as: more efficient lysis (El-Hashemite and Delhanty, 1997), multiplex PCR and fluorescent PCR (Findlay *et al.*, 1995) which allows for more sensitive detection of PCR products. Recent studies in isolated cells and blastomeres are based on the identification of a single *CFTR* mutation (Ray *et al.*, 2002; Vrettou *et al.*, 2002) or on highly polymorphic short tandem repeats (STR) linked to the mutation (Dreesen *et al.*, 2000; Eftedal *et al.*, 2001; Goosens *et al.*, 2003). The most recent alternatives for PGD of cystic fibrosis are sequencing and mini-sequencing methods, with which a wide range of mutations may be detected directly (Bermúdez *et al.*, 2003; Fiorentino *et al.*, 2003).

The aim of the present work was to develop a sensitive and reliable method for the identification of a large number of different mutations in a single procedure. Isolated cells were subjected to a modified and improved primer extension preamplification PCR (I-PEP-PCR) (Dietmaier *et al.*, 1999). The products obtained were used in a multiplex PCR and a fluorescent oligonucleotide ligation assay (OLA) for the identification of normal and mutant loci of the 31 most frequent mutations in Southern Europe (including the 25 most common worldwide). In our hands, the first protocol of PEP-PCR (Zhang *et al.*, 1992) was successful in small clumps of cells (5–30) and in 20 pg of genomic DNA. In order to improve the performance of whole genome amplification in single cells, two different *Taq* polymerases were tested.

Materials and methods

Strategy

To develop a unique PGD system to detect diverse mutations, whole genome amplification was applied to isolated cells, enabling the use of a commercial kit which allows for fluorescent detection of 31 cystic fibrosis mutations. Two different *Taq* polymerases were tested to improve the performance of the preamplification step. The validation of both procedures was based on the height of the alleles detected in the electropherogram. Moreover, the method which yielded the best results was tested in oocytes.

Cell samples

Isolated buccal cells were collected by mouthwashes from three normal individuals, three patients affected by mutations ΔF508, ΔF508/1078delT and ΔF508/3849+10kbC>T and five heterozygous carriers for *CFTR* mutations ΔF508, N1303K, G542X, R347P and 2183AA>G.

Ten matured oocytes remaining unfertilized after IVF, donated by assisted reproductive centres, were available for analysis. Both MII oocytes and 1PBs were studied separately.

All procedures were approved by the Ethics Committees of the participating centres.

Cell isolation

Stringent precautions were followed to guard against contamination during the entire process of isolation, lysis and amplification (Wells and Sherlock, 1998; Thornhill and Snow, 2002). The work was performed in a restricted area in a flow hood with no DNA or PCR product access. The technician wore a gown, surgical mask and gloves, and dedicated pipettes and filter tips were used in the procedure. The reagents used were subjected to filtration, sterilization and UV exposure.

Manipulation was performed using UV-sterilized pulled-glass micropipettes. Buccal cell suspension was diluted in several droplets of sterile phosphate-buffered saline (PBS) with 0.1% polyvinyl alcohol (PVA) to prevent cell sticking, until a single cell was obtained. Buccal cells were checked under stereomicroscope for the presence of a visible nucleus. MII oocytes and 1PBs were separated by digestion of the zona pellucida with either 30 mg/ml trypsin in PBS, pH 7.2, for 2–3 min at 37°C, or with Tyrode's acid solution (pH 2.5). Cells were washed three times in PBS/0.1% PVA and transferred to a 0.2 ml PCR tube. The presence of the cell inside the tube was confirmed.

Cell lysis

One microlitre of sodium dodecyl sulphate 17 μM (Sigma, USA) and 2 μl of PCR-grade proteinase K (125 mg/ml) (Roche, Switzerland) were added to each tube and covered with one drop of light mineral oil (Sigma, USA). The lysis was performed by incubation for 1 h at 37°C, followed by 15 min at 95°C to inactivate the enzyme.

Whole genome amplification in single cells

The lysed cells were subjected to I-PEP-PCR whole genome amplification (Dietmaier *et al.*, 1999) with several modifications: (1) The reaction was performed in 40 μl with five units of *Taq* polymerase, 4 μl of 10× *Taq* buffer, 3.3 μl of a 400 μM solution of totally degenerated 15-mer primers (Zhang *et al.*, 1992) and 2 μl of a mixture of the four dNTPs (2 mM each); (2) Two *Taq* polymerases were used: SuperTaq polymerase (*Taq* A) (HT Biotechnology, UK), specially recommended in single-cell PCR and SuperTaq Plus polymerase (*Taq* B) (Ambion, USA), a mixture of thermostable DNA polymerases with proofreading activity, enabling a high yield of PCR products up to 20 kb. Twenty single cells were amplified with *Taq* A and a further 30 isolated buccal cells and 10 1PB-MII pairs were amplified with *Taq* B; (3) Hotstart was performed keeping the tubes in ice and placing them in a Tgradient (Biometra, Germany) thermal cycler when it reached the required temperature of 80°C; (4) The initial denaturation was performed at 94°C for 7 min and (5) Modified I-PEP-PCR consisted of 50 cycles of 1 min at 94°C, 2 min at 31.5°C, a programmed ramp of 0.1°C per second, 55°C for 4 min and a final step at 68°C for 30 s. Tubes were then cooled down to 4°C. Blank controls of the isolation solutions and PCR reagents were included to check contamination. Tubes were kept at −80°C if necessary.

Cystic fibrosis assay in single cells

Six microlitres of the I-PEP-PCR products were used in an adaptation of the 'Cystic Fibrosis Assay' (Applied Biosystems, USA), a commercially available test for the screening of the most common *CFTR* mutations. It consists of a multiplex PCR in which 15 pairs of primers amplify the regions of the gene which may contain the mutations. Then, a multiplex, fluorescent OLA detects normal and mutant alleles with a complementary probe for each sequence which are ligated with a common probe for every locus labelled with 6-FAM (6-Carboxyfluorescein), TET (tetrachlorafluorescein) or HEX (hexachlorofluorescein). The normal and mutant oligonucleotides have different lengths of pentaethylene oxide tails, with subsequent electrophoretic separation. The mutations assayed are: ΔF508, ΔI507, Q493X, V520F, 1717-1G>A, G542X, G551D, R560T, S459R, S459N and R553X labelled with FAM (blue), 3849+10kbC>T, 3849+4A>G, R1162X, 3659delC, W1282X, 3905insT, N1303K, 1078delT, R347P, R347H and R334W labelled with TET (green) and A455E, 1898+1G>A, 2183AA>G, 2789+5G>A, G85E, 621+1G>T, R117H, Y122X and 711+1G>T labelled with HEX (yellow). The 10 μl of the OLA products of a single cell were additionally ethanol-precipitated for 1 h at −80°C and resuspended in 1 μl of nuclease-free water and a TAMRA (Carboxytetramethylrhodamine) size standard was added. Single cells produced a very small amount of PCR products, so it was necessary to ethanol-precipitate the OLA reaction to concentrate the fragments obtained. The samples were run in an ABI Prism 377 (Applied Biosystems, USA) automated gel electrophoresis. Subsequent analysis was performed with GeneScan and Genotyper softwares (Applied Biosystems, USA). Each allele was identified by its name, fragment size in base pairs and fluorescence intensity in relative fluorescence units (RFU).

Scoring criteria

Detection of an allele has been considered when a single peak was equal to or above 50 RFU in height. A rigorous classification of three categories, based on allele height, was established for evaluating the success and quality of both procedures: peaks below 50 RFU were considered not detected, when the peak ranged from 50 to 250 RFU the detection was considered as low and peaks over 250 RFU were classified as high. Statistical analysis was performed with SPSS software, version 12.5, to compare the results obtained with both *Taq* A and B.

Results

Identification of the normal and mutant alleles of the *CFTR* gene was achieved with both polymerases tested in preamplified DNA of single cells. Since the mutations to be analysed are known, the rest of the peaks of the normal loci can be used as a control of the amplification. No additional unknown mutations were observed after whole genome amplification. Thus the genotype of the cells was determined correctly. Figure 1A shows an electropherogram of a buccal cell carrying mutations ΔF508 and 3849+10kbC>T.

Twenty single buccal cells were analysed using *Taq* A for amplification with PEP-PCR. Preamplification with *Taq* B was performed in 30 single buccal cells. Table I shows the number of cells analysed for the indicated allele, the percentage of cells in each category of classification and amplification rates for both polymerases. From a total of 30 alleles, a mean of 22.3 alleles was identified per cell using *Taq* A, ranging from 15 to 30. In the case of *Taq* B, an average of 26.4 alleles was identified correctly, ranging from 19 to 31, from a total of 31 alleles. The median values for efficiencies of amplification were 80% with *Taq* A and 90% with *Taq* B, both showing statistically significant differences ($P < 0.0001$) in a Wilcoxon test for non-parametric data. Peaks included in the high category (>250 RFU) were used for identifying which *Taq* achieved better results. *Taq* A revealed a median value of 15% of high peaks, while in the case of *Taq* B it was 40%. The difference between both

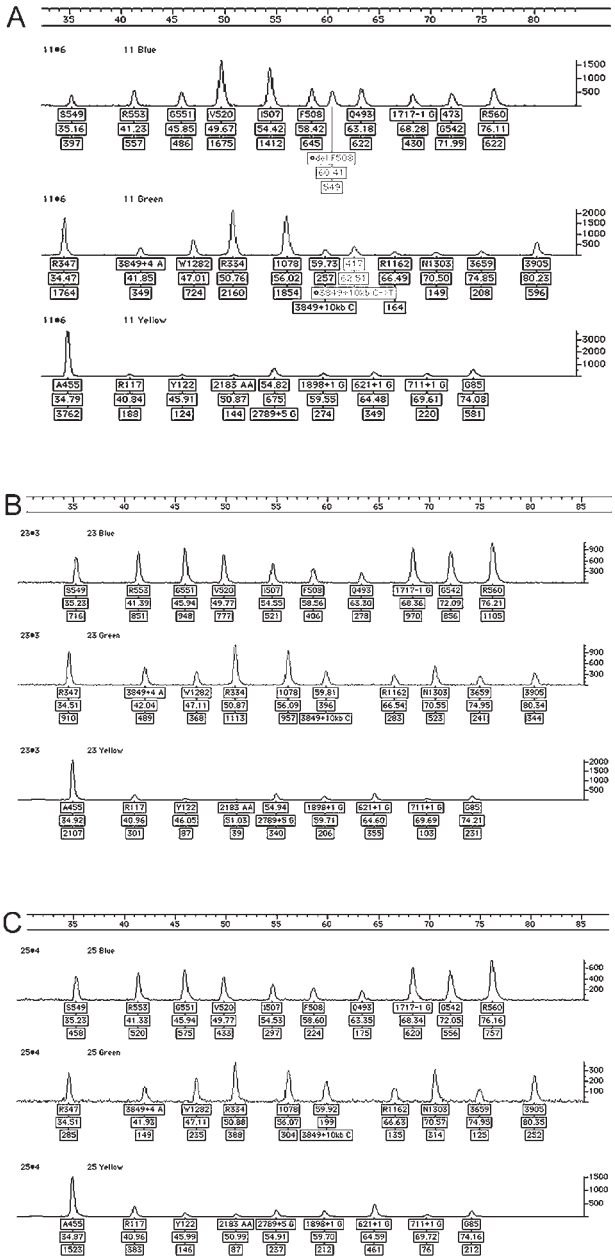

Figure 1. Electropherograms obtained from the analysis of (**A**) a compound heterozygote ΔF508/3849+10kbC>T single cell preamplified with the *Taq* with proofreading activity (*Taq* B), (**B**) a MII and (**C**) 1PB corresponding to Oocyte 2 in Table III. Each allele detected is labelled with three tags corresponding to its name, size (in bp) and height in RFU.

polymerases was also statistically significant ($P < 0.0001$) with the same test.

In the ΔF508 locus, a 30% increase in peaks over 250 RFU was found in both normal and mutant alleles by changing *Taq* A for the proofreading *Taq*, while the amplification rate was similar. In most cases, PEP-PCR with *Taq* B achieved better results and amplification rates than *Taq* A. About 10% heterozygous ΔF508 cells amplified with *Taq* B displayed alleles below 50 RFU, but most of them could be properly identified. A similar result was seen in heterozygous cells for G542X and 3849+10kbC>T. Heterozygous cells for the R347P mutation successfully amplified both alleles with the proofreading *Taq*. Locus A455 showed the highest values in all cells tested. Other loci with high detection were V520, I507, R334 and 1078. On the other hand, amplification in locus 2183AA was extremely low (25% in Protocol A and 33.3% in B), and mutation 2183AA>G was barely detected in only one of seven cases (14.3%). The ADO rate for the assay with *Taq* A was 16.6% because 3 of 18 heterozygous samples generated only one signal. The ADO rate in Protocol B was 13.2% (5/38).

Ten oocytes were also analysed with *Taq* B (Table II), the average of amplification rate of oocytes was 74.5% and in 1PBs 44.5%. The total number of alleles identified in the 10 pairs of 1PBs and MII oocytes is shown in Table III. On average, the alleles detected in 1PBs were 12.9, while in MII oocytes it was 21.7, from a total of 29 normal alleles. Figure 1B and C show electropherograms obtained in the analysis of MII and 1PB pair of Oocyte 2 in Table III, respectively.

The contamination rate was 2.7% (2 of 70 blanks showed amplification).

Discussion

The present work describes an improved whole genome amplification in isolated cells which allows for performing standard laboratory methods for single gene diagnosis with no extensive modifications. PEP-PCR is a reliable method of preamplification because the expected genotype in isolated cells is found and artefacts after PEP were not observed with either of the polymerases chosen. Considering that an increase in denaturing time has beneficial effects on the ADO rate (Piyamongkol *et al.*, 2003), a long initial denaturation step at 94°C for 7 min was performed for ensuring dissociated strands. This option was preferred, instead of increasing the temperature, for avoiding DNA damage. A step of 30 s at 68°C was added (Dietmaier *et al.*, 1999) because it is the optimal temperature of polymerization for both polymerases (according to manufacturers) and longer genetic fragments are obtained.

Amplification rates were calculated considering only peaks over 50 RFU. The protocol with *Taq* B showed a better amplification rate (90%) over *Taq* A (80%), and *Taq* B yielded more results over 250 RFU than *Taq* A (median values of 40 versus 15%). This may be explained by the fact that proofreading activity repairs mistakes during elongation in PCR, permitting high yield as well as longer DNA fragments. The critical choice of the polymerase has been previously reported (Thornhill *et al.*, 2005). Locus R347P reached a 100% amplification rate, while most of the heterozygous loci displayed efficiencies barely over 90%, suggesting that as PCR primers have to be shared between the two alleles, heterozygous samples seem to have less amplification than homozygotes. The ADO rate was evaluated considering heterozygous samples in which one of the alleles was undetected. *Taq* B showed an acceptable ADO rate of 13.2%, similar to other recent reports (Bermúdez *et al.*, 2003; Handyside *et al.*, 2004). Whole genome amplification does not reduce the ADO rate (Wells and Sherlock, 1998), but PEP-PCR was necessary to have a considerable amount of template because of the variety of mutations detected simultaneously with the reactions of multiplex PCR and OLA. OLA detection works with short fragments, which at first should be less inclined to experience ADO (Piyamongkol *et al.*, 2003).

Only in the case of allele 2183AA and its mutation 2183AA>G, as a result of the failures of amplification accumulated in this locus, would PGD of this mutation not be advisable. The loci with amplification rates below 85% (3849+10kbC>T, N1303K, 2789+5G>A and 711+1G>T) should be studied more in-depth, preferably analysing fresh oocytes. The rest of the loci showed

Table I. Percentages of single buccal cells observed in each category

Allele	*Taq* A					*Taq* B				
	Number of cells	% Cells			Amplification rate (%)	Number of Cells	% Cells			Amplification rate (%)
		n.d. < 50	Low 50–250	High > 250			n.d. < 50	Low 50–250	High > 250	
S549	20	25	60	15	75	30	13.3	46.7	40	86.7
R553	20	15	65	20	85	30	6.7	46.7	46.7	93.3
G551	20	15	70	15	85	30	10	46.7	43.3	90
V520	20	5	40	55	95	30	0	13.3	86.7	100
I507	20	5	45	50	95	30	0	26.7	73.3	100
F508	20	10	80	10	90	30	10	50	40	90
dF508 (*)	12	25	66.7	8.3	75	19	10.5	52.6	36.8	89.5
Q493	20	10	75	15	90	30	10	56.7	33.3	90
1717-G1	20	25	60	15	75	30	6.7	46.7	46.7	93.3
G542	20	20	65	15	80	30	10	50	40	90
G542X (*)	2	50	50	0	50	7	14.3	85.7	0	85.7
R560	20	20	50	30	80	30	6.7	43.3	50	93.3
R347	20	5	70	25	95	30	0	16.7	83.3	100
R347P (*)	–	–	–	–	–	3	0	33.3	66.7	100
3849 + 4A	20	15	55	30	85	30	3.3	50	46.7	96.7
W1282	20	20	40	40	80	30	13.3	30	56.7	86.7
R334	20	10	75	15	90	30	0	13.3	86.7	100
1078	20	25	60	15	75	30	0	13.3	86.7	100
1078 del T (*)	2	0	100	0	100	–	–	–	–	–
3849 + 10kbC	20	20	65	15	80	30	3.3	56.7	40	96.7
3849 + 10kbC > T (*)	–	–	–	–	–	2	50	0	50	50
R1162	20	30	60	10	70	30	13.3	66.7	20	86.7
N1303	20	65	30	5	35	30	16.7	76.7	6.7	83.3
N1303K (*)	4	50	50	0	50	–	–	–	–	–
3659	20	40	55	5	60	30	10	70	20	90
3905	20	20	60	20	80	30	10	33.3	56.7	90
A455	20	0	0	100	100	30	0	0	100	100
R117	20	20	40	40	80	30	10	36.7	53.3	90
Y122	20	45	35	20	55	30	6.7	70	23.3	93.3
2183AA	20	75	20	5	25	30	66.7	30	3.3	33.3
2183AA > G (*)	–	–	–	–	–	7	85.7	14.3	0	14.3
2789 + 5G	20	15	45	40	85	30	26.7	33.3	40	73.3
1898 + 1G	20	45	55	0	55	30	33.3	46.7	20	66.7
621 + 1G	20	5	50	45	95	30	3.3	26.7	70	96.7
711 + 1G	20	80	15	5	20	30	16.7	76.7	6.7	83.3
G85	20	30	50	20	70	30	6.7	53.3	40	93.3
Median (**)				15	80				40	90

n.d. = not detected.

*Heterozygous loci.

**Statistically significant differences ($P < 0.000$) are shown between results for each polymerase (15 versus 40 and 80 versus 90). Median values were calculated since the distribution was not normal.

Table II. Efficiencies of amplification using *Taq* B in Mll oocytes and first polar bodies

Allele	Amplification rate (%)	
	MII	1PB
S549	70	60
R553	80	60
G551	80	60
V520	100	50
I507	100	30
F508	100	20
Q493	80	20
1717-1G	70	60
G542	70	60
R560	70	60
R347	80	70
3849 + 4A	80	60
W1282	80	30
R334	80	70
1078	70	70
3849 + 10kb C	80	60
R1162	40	60
N1303	70	30
3659	30	60
3905	80	30
A455	100	80
R117	90	30
Y112	50	10
2183AA	40	20
2789 + 5G	70	20
1898 + 1G	80	40
621 + 1G	90	30
711 + 1G	40	10
G85	90	30
Mean	74.5	44.5

Table III. Number of alleles detected in oocytes

Oocyte	MII	1PB
1	28	18
2	28	29
3	25	7
4	26	11
5	24	0
6	12	12
7	20	18
8	12	20
9	27	14
10	15	0
Mean	21.7	12.9

efficiencies of amplification suitable for use in clinical application. The 'Cystic Fibrosis Assay' (Applied Biosystems, USA) commercial kit is used in molecular diagnostic centres for the screening of *CFTR* mutations in cystic fibrosis patients and for carrier identification. Genomic DNA from peripheral blood showed low fluctuations in the height of the alleles due to differences in the efficiencies of amplification for each locus and these variations were increased in preamplified single cells. The possibility of optimization of the kit is rather limited, but the efficiencies of amplification obtained in single cells were very satisfactory in the great majority of alleles.

Controls against contamination are critical when using whole genome amplification, since contaminating cells or amplicons can be over-amplified rather than the assayed cell and thus lead to misdiagnosis. It is a matter that can be dealt with by using severe measures to prevent it and by the application of techniques which detect it (i.e. STRs). Up to six aliquots of each PEP-PCR product can be analysed simultaneously and this technique allows for additional amplification of STRs of the *CFTR* gene (data not shown) for the detection of contamination and ADO. According to our experience, the phenomenon of slippage, reported in the amplification of preamplified repetitive sequences (Foucault *et al.*, 1996; Wells *et al.*, 1999) did not occur when using the *Taq* with proofreading activity (*Taq* B), as mistakes are corrected when using this polymerase.

The present study has been performed under standard electrophoretic conditions for genomic DNA in an ABI Prism 377 sequencer. The step of precipitation should be avoided as varying results were obtained in different aliquots assayed from the same PEP product. Results could be improved by using more sensitive apparatus, such as an ABI Prism 3100. Variations in injection time or voltage could make detection of certain fragment sizes and weaker fluorescent dyes better. Additionally, electrophoresis is performed simultaneously in 16 capillaries, and all cells tested can be analysed in a single run.

Buccal cells are not completely representative, but they are easier to obtain and can be selected from carriers of mutations in the gene under study. Spare embryos and oocytes from finished IVF cycles are in limited availability and have variable, often suboptimal genetic quality. Oocytes were processed at least one day after retrieval, which could explain the better results of MII compared to 1PB, where degeneration by apoptosis may have started earlier than in the oocyte (Gitlin *et al.*, 2003). Several 1PBs were extensively degraded and completely failed to amplify (1PB numbers 5 and 10 in Table III) and in only one case (1PB number 2) were all normal alleles detected. This oocyte ageing could also explain the difference between the oocytes and the buccal cells. Ageing produces nicks in the DNA with detrimental amplification results.

Handyside *et al.* (2004) have recently described isothermal amplification and its possible application in PGD. Their technique needs overnight incubation (about 16 h), while our method of preamplification lasts 6 h less, in which molecular diagnosis could be performed. Saving time is crucial in the case of PGD in blastomeres, when only 32 h are available for diagnosis before transfer. The entire process (lysis, I-PEP-PCR, multiplex PCR, OLA, electrophoresis and analysis) takes about 30 h, so it would be compatible with 1PB analysis and Day +2 embryo replacement in the case of single gene defects maternally inherited. Blastomere analysis should also be possible since embryo transfer can be carried out the third or fourth day after oocyte retrieval (Durban *et al.*, 2001).

This work represents the first description of the adaptation for PGD of a commercial test for the screening of cystic fibrosis mutations with fluorescent detection. The use of the commercial assay allows for the use of a single procedure for the identification of the 31 most common mutations, avoiding the need for having a different protocol for every mutation tested. Therefore, it could be a very useful strategy for its application in centres offering PGD. The method described here directly detects the mutation as in minisequencing strategies (Bermúdez *et al.*, 2003; Fiorentino *et al.*, 2003). However, it is important to note that these procedures are not exempt from primer design for every different mutation tested. This procedure would be applicable to 80% of couples worldwide carrying a *CFTR* mutation. This data is close to the 87% described by Dreesen *et al.* (2000) in the Dutch population with their microsatellite approach. A global strategy including the present technique and STR analysis for additional indirect diagnosis (when informative) and detection of contamination and ADO would be a very powerful approach.

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