GENETICS

Direct Comparison of Detection Systems Used for the Development of Single-Cell Genetic Tests in Preimplantation Genetic Diagnosis

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Purpose: Single-cell polymerase chain reaction (PCR) requires efficient amplification and accurate detection. We compare the accuracy of heteroduplex, fluorescent-fragment, and fluorescent single-strand conformation polymorphism (F-SSCP) analysis as detection systems for analysis of a PCR assay developed for preimplantation genetic diagnosis.

Methods: A single-cell, fluorescent multiplex PCR assay was developed for the cystic fibrosis $\Delta F508$ mutation and the short tandem repeat, D21S11. Detection systems were compared by analyzing blinded PCR products.

Results: Amplification rates for cystic fibrosis were 89% by heteroduplex and 91% by fragment analysis, while it was 72% for D21S11 by fragment analysis. No difference in allele dropout was detected for cystic fibrosis by any method (2%). Overall accuracy was high, >97%, although SSCP was the least accurate.

Conclusions: Heteroduplex and fragment analysis proved equal in the diagnosis of a single amplified locus. We determined that fragment analysis allows maximal accuracy of detection and permits analysis of a second loci, controlling for DNA contamination and allelic dropout.

KEY WORDS: allele dropout; fluorescence multiplex PCR; preimplantation genetic diagnosis; short tandem repeats.

INTRODUCTION

Multicolor FISH is used universally to screen cells for chromosomal abnormalities. This technique is employed in more than half of all preimplantation genetic diagnosis (PGD) cases that are performed for older women (>35 years old) requesting aneuploidy screening (1). In contrast, the diagnosis of each single-gene defects requires the development of a polymerase chain reaction (PCR) protocol designed specifically for the mutation of interest. This is a major limitation, since any new protocol must be rigorously tested at the single-cell level prior to its clinical use. Development of PGD tests for the most common genetic disorders can be easily justified. However, many diseases are caused by a spectrum of mutations (e.g., cystic fibrosis (CF) and beta-thalassemia), with some occurring very rarely within the general population. Since developing a new assay involves a considerable investment of time and resources (2), it is impractical for PGD centers to standardize protocols case by case. Therefore, advancements in the diagnosis of single-gene defects have all stemmed from the necessity to make each test rapid, accurate, and cost-effective, while also offering it to as large a patient base as possible. In recent years, the literature has been full of reports promoting the advantages of multiplex PCR

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(3), fragment analysis (4,5), and single-strand conformation polymorphism (SSCP) (6,7) in the detection of genetic disorders for PGD. These papers describe the standardization of PCR protocols for vastly different disease mutations, but have not made a direct comparison between detection systems.

Owing to the sensitivity of the technique, PCR can be problematic. PCR for the genotyping of single cells is a distinct technique compared to that of PCR on purified DNA. Complications include the rate of amplification failure, allelic dropout (ADO), chromosomal mosaicism, and DNA contamination. PCR protocols have been adapted to try to address most of these issues but cannot correct for inherent biological defects such as chromosomal mosaicism. However, linked polymorphic markers, e.g., short tandem repeats (STRs), can be included in multiplex PCR protocols as additional controls for ADO and DNA contamination. It has been proposed that a contributing factor for high ADO rates could be the low detection sensitivity offered by standard electrophoresis and ethidium bromide staining (8). Therefore, many existing single-cell PCR protocols have been modified to include fluorescent primers for fluorescent fragment analysis (fragment analysis) (4,5,8); a system reported to be 1000 $\times$ more sensitive than standard gel electrophoresis (9).

It is clear that not all PCR methodologies or DNA detection techniques are equivalent. The advantages provided by fragment analysis, SSCP, and standard gel electrophoresis must be weighed against the accuracy of each test and the cost-effectiveness of using the method routinely for PGD. Therefore, we set out to compare the reliability of these three techniques in the analysis of a single-genetic test developed specifically for PGD. In blind studies, normal and CF-carrier single lymphocytes as well as CF-affected genomic DNA were subjected to a multiplex PCR assay to detect the CF Δ F508 mutation and the polymorphic tetranucleotide short tandem repeat (STR), D21S11. All PCR products were labeled with fluorescent primers and the fragments subjected to heteroduplex analysis using standard gel electrophoresis and visualized directly on an ALFexpress DNA sequencer for fragment analysis or fluorescent-SSCP (F-SSCP). We discuss our results in terms of how truly the detection system reflects the PCR product i.e. accuracy, but do include analysis involving the efficiency of PCR i.e. correct diagnosis and consider issues surrounding the development of new genetic tests for routine PGD.

MATERIALS AND METHODS

Isolation of Single Cells

Blastomeres were from research embryos donated by a couple undergoing PGD for CF. Single human lymphocytes and blastomeres were collected and isolated according to the protocols outlined by Blake *et al.* (10). To promote efficient PCR amplification, individual cells were lysed by using either an alkaline lysis buffer (11) or Proteinase K and sodium didecyl sulfate buffer system (12). Previous studies have indicated no significant advantage to using alkaline lysis over Proteinase K and sodium didecyl sulfate buffer system (10).

PCR Conditions

To screen for CF mutations we used nested primer sequences that flanked the Δ F508 mutation in exon 10 (13) of the cystic fibrosis conductance regulator gene (CFTR). DNA primers were designed to amplify D21S11 (forward: 5-gtgagtcattcccccaagtg-3; reverse: 5-gttgtattagtcaatgttctcc-3), a STR from a polymorphic locus on chromosome 21. Both CF inner forward and D21S11 forward primers were labeled with CY5 fluorescent dye. All primary round PCR reactions were performed using the same experimental conditions. Reaction mixes were in a total volume of 50 μ L (lymphocytes) or 30 μ L (blastomeres) and included a final concentration of 100 μ M of each dNTP, and either 1) 1 $\times$ neutralizing buffer (900 mM Tris-HCl [pH 8.3], 300 mM KCl, 200 mM HCl), 1 $\times$ potassium-free PCR buffer (25 mM MgCl₂, 100 mM Tris-HCl [pH 8.3]) for lymphocytes or 2) 1 $\times$ Qiagen PCR (200 mM Tris-HCl [pH 8.4], 500 mM KCl, 15 mM MgCl₂) for blastomeres, and 1 unit of Taq polymerase (Qiagen, Mississauga, Ont., Canada) and the correct concentration of the appropriate primer set combination (0.2 μ M of each CF outer primers and 0.4 μ M of D21S11 primers). The thermocycler (PTC-200 DNA engine, MJ Research, Watertown, MA, USA) was programmed to denature the DNA for 4 min at 96°C; followed by 18 cycles at 96°C for 1 min, 40°C for 45 s, 72°C for 45 s, and a final elongation step of 72°C for 10 min.

For the second round, 2 μ L of the first round products were added to 30 μ L reaction mixes containing a final concentration of 1 $\times$ Qiagen PCR buffer, 1 unit of Taq polymerase (Qiagen), and either 200 μ M of dNTP and 0.8 μ M of CF inner primers or 100 μ M of dNTP and 0.4 μ M of D21S11 primers. CFTR exon

10 and D21S11 were amplified separately using the same amplification conditions as described for the primary round, with the following exceptions: 1) CF PCR products were initially denatured at 96°C for 10 cycles, with an annealing temperature of 50°C for 1 min and an elongation step of 72°C for 1.3 min, followed by an additional 20 cycles with the denaturing temperature at 94°C and 2) D21S11 primers were annealed at 58°C for 28 cycles.

Analysis of PCR Products

For our purpose, “correct diagnosis” is the percentage of samples that produce the expected genotype that is related to the efficiency of PCR (but not including the cells that failed to amplify), whilst “accuracy” is how faithfully the detection system profiles each amplified product and is independent of PCR efficiency.

To accurately assess the reliability of these three detection techniques, we performed all PCR experiments blind. Samples were coded such that individuals assigning diagnoses were not aware of the expected number of single cells or the relative ratio of normal, carrier, or affected samples.

CF Heteroduplex Analysis

PCR aliquots of 8 μ L were mixed with either 6 μ L of previously amplified known homozygous normal or homozygous CF-affected DNA. The samples were denatured at 95°C for 4 min and then allowed to cool to room temperature. Homo- and heteroduplex double-stranded DNA samples were resolved on a 10% bis/acrylamide gel and visualized after ethidium bromide staining (Fig. 1(A)).

Fragment Analysis

The fluorescently labeled amplified DNA was visualized by running products on an ALFexpress sequence analyzer (Amersham Pharmacia Biotech). The equivalent of 1 μ L of CF (Fig. 1(B)) and 5 μ L of D21S11 PCR products with 4 μ L loading dye were denatured for 3 min at 90°C prior to the samples being loaded onto a 9% bis/acrylamide denaturing gel. DNA was detected as fluorescent peaks using the instruments packaged computer software. A fluorescently labeled 50–500 bp marker (Amersham Pharmacia Biotech) was loaded onto each gel and used to size peak fragments.

Fig. 1. CF diagnosis by conventional and fluorescent detection systems. Normal (NN) and carrier (Δ N) single cells, as well as CF-affected ($\Delta\Delta$) DNA were screened for the CF Δ F508 mutation, using our multiplex PCR assay. Aliquots of each amplified sample were analyzed by standard gel electrophoresis/heteroduplex (A), fragment (B), and F-SSCP (C) analysis (M, Hae III marker; bp, base pair).

F-SSCP

PCR aliquots (2 μ L) of amplified CF DNA were mixed with 4.5 μ L of both formamide and loading dye, denatured at 95°C for 4 min and loaded onto an ALFexpress 9% non-denaturing bis/acrylamide gel. Single-stranded DNA was resolved over 200 min at 800 V, with the temperature set to 15°C (Fig. 1(C)).

RESULTS

CF Mutational Analysis and D21S11 Allele Detection in Single Cells

Initially, PCR conditions were standardized using fluorescent PCR and fragment analysis. Amplification of D21S11 was attempted using a hemi-nested primer approach; however, higher amplification was achieved using conditions described earlier (10). With fragment analysis, 36 PCR cycles was sufficient to produce amplification rates >90% for CF and D21S11 in singleplex reactions (data not shown). However, we needed to compare the accuracy of fluorescent PCR with heteroduplex analysis, therefore it was necessary to develop a nested-PCR assay. Using only 18 cycles for the primary and either 30 and 28 for the secondary amplification of CF and D21S11, respectively, the overall amplification rate, in carrier cells, for each of these loci was >90% and the correct diagnosis was 100% (data not shown). This assay was subsequently used for all blinded PCR experiments.

Single-Cell PCR Analysis by Heteroduplex Analysis, F-SSCP, and Fragment Analysis: Blinded Study

Using the multiplex PCR assay for CF and D21S11, a total of 120 single lymphocytes, either normal or CF-carrier, and 20 CF-affected DNA samples were genotyped by heteroduplex analysis and fragment analysis (Table I). The data show affected genomic CF DNA (starting material equivalent to ~16–20 single cells) was accurately detected and correctly diagnosed in all 20 samples (100%) by both techniques and that the diagnosis was verified in each case by the coamplification of the unique D21S11 allele profile. For single-cell diagnosis, both heteroduplex analysis and fragment analysis proved to be highly accurate in detection rate (99 and 100% respectively) with one inaccuracy in the detection of a normal cell with heteroduplex analysis. Samples that were misdiagnosed, due to ADO (2% of CF-carrier cells), were identified by both detection

methods. Fragment analysis was shown to be slightly more sensitive than standard gel electrophoresis. Two CF normal cells, scored as failed amplification by heteroduplex analysis, were correctly genotyped by fragment analysis, therefore increasing the overall amplification rate for single cells from 89 to 91%.

Fragment analysis was used successfully to detect D21S11 alleles in 72% of the single cells tested with 10% ADO and in 100% of CF-affected DNA with 5% ADO (Table II). Even though all experiments were performed under identical PCR conditions there was a variation in the ADO rate between experiments. A total of five out of the seven ADOs for D21S11 were produced by a single-cell population, three of which were detected in a single experiment. Two of the samples exhibiting ADO of one D21S11 allele, one normal and another CF-affected, could be genotyped because of amplification of an allele unique to these samples. This permitted 93% (99/107) of total amplified samples (single lymphocytes plus genomic DNA) to be correctly diagnosed.

CF diagnosis by F-SSCP proved to be less accurate. After standardization of running conditions, CF normal, CF-carrier, and CF-affected samples could be easily distinguished after F-SSCP (Fig. 1(C)). The accuracy of the technique was determined by using previously amplified products, which gave results with fragment analysis, in a blind experiment. Similar to the methods mentioned earlier, tubes were coded and the diagnoses performed without prior knowledge of the relative number of CF normal, CF-carrier, and CF-affected samples. Table I shows the results of these experiments. Out of a total of 129 samples tested using SSCP (single lymphocytes plus genomic DNA), 126 were accurately detected (98%) and 125 (97%) were correctly diagnosed. One of the four samples showing misdiagnoses by F-SSCP was confirmed as displaying ADO by fragment analysis. In this case, the genotype was incorrect but the actual accuracy of F-SSCP was no different than for fragment analysis. Of the remaining three samples, one normal cell was scored as affected while two other normal cells were identified as carriers. These results are discordant with the genotypes assigned to these samples by both heteroduplex analysis and fragment analysis and, therefore, affect the accuracy and correct diagnosis rate of F-SSCP.

The multiplex PCR assay was developed with the intent that it would be used for future PGD. Thus, the STR was included in the test as a means to control for DNA contamination. Blastomeres from spare embryos, donated to research from a couple undergoing PGD for CF, were screened for the Δ F508 mutation

Table I. Blind Study: Amplification Efficiency and Accuracy of CF Diagnosis by Conventional Heteroduplex Analysis, Fragment Analysis, and SSCP

Sample type	Genotype	Heteroduplex analysis				Fragment analysis				SSCP		
		Amplification	ADO	Accuracy of detection	Correct diagnosis	Amplification	ADO	Accuracy of detection	Correct diagnosis	ADO	Accuracy of detection	Correct diagnosis
Lymphocyte	NN	52/60 (87%)	N/A	51/52 (98%)	51/52 (98%)	54/60 (90%)	N/A	54/54 (100%)	54/54 (100%)	N/A	51/54 (94%)	51/54 (94%)
Lymphocyte	ΔN	55/60 (92%)	1/55 (2%)	55/55 (100%)	54/55 (98%)	55/60 (92%)	1/55 (2%)	55/55 (100%)	54/55 (98%)	1/55 (2%)	55/55 (100%)	54/55 (98%)
Total (single lymphocytes)		107/120 (89%)	2%	106/107 (99%)	105/107 (98%)	109/120 (91%)	2%	109/109 (100%)	108/109 (99%)	2%	106/109 (97%)	105/109 (96%)
DNA (100pg)	ΔΔ	20/20 (100%)	N/A	20/20 (100%)	20/20 (100%)	20/20 (100%)	N/A	20/20 (100%)	20/20 (100%)	N/A	20/20 (100%)	20/20 (100%)

Note. “Accuracy” is defined as how faithfully the detection system profiles each amplified product and is independent of PCR efficiency. “Correct diagnosis” is defined as the percentage of samples that produce the expected genotype and is related to the efficiency of PCR.

Table II. Blind Study: Amplification Efficiency and Correct Diagnosis Rates for D21S11 Using Fluorescent Fragment Analysis

Sample type	Sample size	Genotype	D21S11 Fluorescent		
			Amplification	ADO	Correct diagnosis
Lymphocyte	60	NN	39/60 (65%)	7/39 (18%)	33/39 ^a (85%)
Lymphocyte	60	Δ N	48/60 (80%)	2/48 (4%)	46/48 (96%)
Total (single lymphocytes)	120		87/120 (72%)	9/87 (10%)	79/87 (91%)
DNA (100pg)	20	$\Delta\Delta$	20/20 (100%)	1/20 (5%)	20/20 ^a (100%)

^aTwo samples, one normal and another CF affected, exhibited ADO of one D21S11 allele. Nonetheless, because of amplification of an allele unique to these samples, each sample could be correctly genotyped.

and D21S11 profiles. In this case, all CF normal embryos were used for embryo transfer. Figure 2(A) illustrates the CF-carrier profiles of each parent and the examples of CF-carrier and CF-affected embryos.

The genotypes of the spare embryos can be distinguished from each other, and shown to be related to the parents, by the analysis of the inherited STR alleles (Fig. 2(B)).

Fig. 2. CF diagnosis and D21S11 alleles in single cells. Parental and embryo profiles were assessed by using our multiplex assay to screen single CF-carrier lymphocytes (maternal and paternal) and blastomeres for the CF Δ F508 mutation (A) and D21S11 (B). Carrier (Δ N) and CF-affected ($\Delta\Delta$) single cells were genotyped by running samples on a fluorescent DNA sequencer for fragment analysis (b.p., base pair).

DISCUSSION

We developed a reliable fluorescent multiplex PCR assay to screen normal, carrier, and CF-affected samples for the CF $\Delta F508$ mutation and the STR, D21S11. The CF $\Delta F508$ mutation is caused by a 3-bp deletion in exon 10 of the CFTR gene while the STR is a tetranucleotide repeat. By amplifying these two DNA fragments it was possible to assess both the reliability of multiplex PCR and the accuracy with which small base pair differences can be resolved by fragment analysis and F-SSCP. We distinguish the accuracy of detection from the percentage of correct diagnoses because the aim of this paper is to assess the capability of each detection system. For example, ADO would not be an inaccuracy of detection as is related to the efficiency of PCR but would however be considered a misdiagnosis. Obviously, in the clinical situation the number of embryos diagnosed correctly are of paramount importance so it is necessary to develop PCR protocols allowing maximal efficiency.

While no single protocol eliminates ADO completely, Findlay *et al.* (8) was the first to detail the advantage of fragment analysis for reducing the rate of ADO by distinguishing the difference between true ADO and preferential amplification of a specific allele. When applied to single-cell analysis, the ADO rate was quoted to be as low as $\sim 4\%$. In this study, and others like it, ADO rates are calculated from amplification of known heterozygote cells. In our own experience, working with the ALFexpress DNA sequencer, we found the machine's "autoscaling" option can influence the size of background peaks if the scale of the *X* axis is altered (i.e. set to a scale range of <150 bp). It is possible that the operator can introduce bias into the experiment by having prior knowledge of the cell type, scoring a true ADO as preferential amplification due to nonspecific background. For this reason, in each of our experiments the *X* axis was set to produce flat background lines, and experiments were performed blind to avoid possible bias. We found no difference in the ADO rate (2%) between CF samples (Table I) analyzed by either heteroduplex analysis or fragment analysis. Thus, in our carrier cells, there was no preferential amplification detected by fragment analysis that was also not correctly genotyped by conventional heteroduplex analysis. The total ADO rate for the STR in our samples (single lymphocytes plus genomic DNA) was 9% (Table II).

Multiplex PCR is another tool used to detect ADO by the inclusion of linked polymorphic markers. This approach has been used as a control for ADO in

single-cell diagnosis of CF (14–16), beta-thalassemia (15), and adenomatous polyposis coli (7). In our case because CFTR and D21S11 exist on different chromosomes, the STR acts as a control for DNA contamination rather than a control for ADO. The inclusion of amplification of one STR in the PCR reaction does not eliminate the possibility of all external DNA contamination (i.e. if the contamination had a similar STR profile to the parental alleles) but does reduce it. Based on a model for controlling misdiagnosis, for a recessive disease with a linked marker, 0.44% of affected embryos would be classified as unaffected compared to 5.8% when the decision is based on disease alleles alone (17).

We used our assay to test spare embryos donated to research by a couple undergoing PGD for CF. Parental STRs were identified using the multiplex PCR assay to screen single lymphocytes. These STR profiles were then compared with the D21S11 alleles amplified from each of the isolated blastomeres. For those samples that showed amplification of the STR, each allele could be identified as having been inherited from either the mother or father (Fig. 2). Therefore, in none of the samples tested was there any evidence of DNA contamination. The significance of this result is important since the presence of contaminating DNA would nullify any genetic result derived from CF genotyping.

SSCP separates DNA by base pair length as well as base pair composition so can be used to screen DNA fragments for both polymorphisms and point mutations (18). After comparing fragment and F-SSCP analysis, we determined an accuracy of detection for CF alleles, from all amplified samples, of 100 and 98% respectively. A sample misdiagnosed by fragment analysis, due to ADO, was similarly genotyped by F-SSCP. There were, however, several normal samples that were misdiagnosed by F-SSCP giving total rates of correct diagnosis of 99% for fragment analysis and 97% for F-SSCP. One normal sample was genotyped as affected. In this case, the discrepancy can be explained by possible lane-to-lane variation. A slight shift in the migration of DNA can, in this instance, make a normal sample appear affected. Two other samples were diagnosed as carriers that had been correctly genotyped by heteroduplex and fragment analysis as normal with no evidence of DNA contamination. Since SSCP profiles are dependent on the secondary structure of DNA, it is most likely that inefficient denaturation of samples prior to gel loading resulted in residual secondary structure, producing a DNA profile for these samples that

closely resemble carrier cells. Therefore, in comparison with fragment analysis, F-SSCP proved to be unreliable for single-cell analysis.

Generally cleaved embryos that have undergone PGD are preferably transferred within a day or two of biopsy (19–21). These criteria set strict limitations on the length of time permitted for biopsy and diagnosis. During our initial investigation, we developed a highly efficient single round (36 cycle) CF ($\Delta F508$) PCR assay using fragment analysis (unpublished data). However, in order to investigate the accuracy of heteroduplex, fragment, and F-SSCP analysis with the inclusion of a control for DNA contamination, it was necessary to develop a nested PCR assay. Using our PCR protocol for CF, the most rapid detection method was by heteroduplex analysis ($\sim 3\frac{1}{2}$ h) that had been routinely used for PGD (7,13). Standard gel electrophoresis has also been applied clinically for the analysis of STRs (3,15,22). In contrast, F-SSCP diagnosis could only be made in 5 h which is almost 2 h longer than for heteroduplex analysis. Diagnosis, using the standardized protocol with fragment analysis could be made in just under 5 h. This time can actually be reduced by 30 min because detection by fragment analysis requires only 22 cycles of amplification for CF in the second round of PCR (unpublished data).

Developing a PGD program requires a considerable initial investment in expensive equipment (2). For single-gene defects, like CF ($\Delta F508$) or SMA (telomeric deletion), a single-cell genetic test can be developed for standard gel electrophoresis (Bio-rad electrophoresis unit: ~ 500 US\$) or fragment analysis (automated fluorescent DNA sequencer: $\sim 100,000$ US\$). However in both methods, the equipment can be used for diagnosis of many PCR products after each test has been developed. Standard gel electrophoresis is an affordable and rapid technique but led to the misdiagnosis of one sample in our blinded experiment. Whilst fragment analysis is more expensive we found it gave the most accurate diagnosis for CF. Correct genotyping is of paramount importance to clinical PGD. This coupled with the fact that fragment analysis allows to differentiate small base pair differences in a multiplex PCR assay and can give a diagnosis of products in just over 4 h, lead us to conclude that it is the method with the most reliability and flexibility.

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