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Application of Hot Start PCR Method in PCR-based Preimplantation Genetic Diagnosis

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Purpose: To determine a method to improve the efficacy and accuracy of preimplantation genetic diagnosis (PGD) - polymerase chain reaction (PCR), we compared hot start PCR and conventional multiplex nested PCR.
Materials and Methods: This study was performed with single lymphocyte isolated from whole blood samples that were obtained from two couples with osteogenesis imperfecta (OI). We proceeded with conventional multiplex nested PCR and hot start PCR in which essential reaction components were physically removed, and we compared the amplification rate, allele dropout rate and nonspecific products. Afterward, we used selective method for PGD.
Results: In the two couples, the respective amplification rate were 93.5% and 80.0% using conventional multiplex nested PCR and 95.5% and 92.0% using hot start PCR. The respective mean allele dropout rates for the two couples were 42.0% and 14.0% with conventional multiplex nested PCR and 36.0% and 6.0% with hot start PCR.
Conclusion: The results demonstrate that the hot start PCR procedure provides higher amplification rates and lower allele dropout rate than the conventional method and that it decreased the nonspecific band in multiplex nested PCR. The hot start method is more efficient for analyzing a single blastomere in clinical PGD.

Key Words: Hot start PCR, Nested PCR, Preimplantation genetic diagnosis

Introduction

Preimplantation genetic diagnosis (PGD) is a genetic analysis that uses a single blastomere before the embryo transfer step of in vitro fertilization (IVF). Advances in molecular biology and IVF have enabled the use of PGD. Previous reports on the use of PGD-PCR related to the Y-specific DNA amplification on the first PGD-PCR cycle, originated in England in 1990.[1] Recently advanced assay techniques have provided the opportunity to improve the diagnosis of single gene disorders. In particular, PCR has become a highly efficient technique that can rapidly diagnose a variety of single gene defects.[2,3] However, PCR using a single cell cause errors in diagnosis, such as allele dropout because of a low concentration of DNA.[4]

The Taq polymerase included with PCR regents has especially high activity at 37℃.[5] In front case, the researchers may obtain a result in which they fail to amplify the correct band or get to obtain nonspecific bands because of a high mismatch with primers. Thus, fine-tuning PCR conditions can be an intensive process that is susceptible to all the problems of conventional PCR.[3]

The hot start PCR method has been reported to decrease the amount of nonspecific products and primer artifacts.[6] In brief,

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activation of the polymerase occurs after the complete denaturation of DNA.[7] PGD is generally performed with multiplex nested PCR that detects several polymorphisms in a single blastomere, and hot start PCR may therefore have some advantages. There have been a few reports on the hot start PCR method for PGD.[8] However, no reports have compared hot start PCR with conventional multiplex nested PCR in for PGD.

We conducted a study comparing hot start PCR, in which essential reaction components were removed, with conventional multiplex nested PCR. We presumed that manual hot start PCR would provide optimal PGD-PCR conditions with a single blastomere on account of the effective amplification of the target gene, which requires a PGD.

Materials and Methods

1. Study samples

For this study, we used whole blood samples donated by two couples with osteogenesis imperfecta (OI) who visited Seoul National University Hospital. We isolated single lymphocytes from each blood sample using the method outlined below. One of the couples underwent a PGD at a later time.

In the first couple (family 1), both partners had a causal mutation. The female partner carried a mutation in the *COL1A1* gene (c.3470 G>A), and the male partner carried a mutation as well (IVS15+5 G>A). In the second couple (family 2), the female partner had the causal mutation. The mutation was in the *COL1A2* gene (c.3803_3805dup). The mutations of these couples were diagnosed in the other institution (Labgenomics clinical laboratories, Sungnam, KR), where we received related materials.

2. Isolation of single lymphocytes

Whole blood samples were collected in heparinized tubes. Three milliliters of blood was layered onto Histopaque-1077 (Sigma, St. Louis, MO, USA) and centrifuged at 400 g for exactly 30 min at room temperature. The plasma layer was carefully discarded, and the mononuclear cell fraction was aspirated into a clean conical centrifuge tube. The layer was washed twice with Dulbecco's phosphate-buffered saline (PBS). The cell pellet was mixed in 1 mL of Roswell Park Memorial Institutes (RPMI) medium (Gibco, Rockville, MD, USA) containing 10% dimethyl sulfoxide (Merck, Rahway, NJ, USA) and was then frozen at -20℃ until use. For the isolation of single lymphocytes, an aliquot of the cell suspension was thawed and 20 μL was added to 4 mL RPMI medium, supplemented with 10% serum substitute supplement (Irvine Scientific, Santa Ana, CA) in a culture plate. Single lymphocytes were aspirated using a pasteur pipette under an inverted microscope and transferred to 0.2 mL PCR tubes containing 3 μL alkaline lysis buffer (ALB), which were then centrifuged, and heated at 65℃ for 10 min.

3. Conventional multiplex nested PCR

The methods of conventional multiplex nested PCR were modified from Girardet et al.[9] Some of the primers have been previously described in Lab genomics clinical laboratories, while others had to be specially designed based sequences of Primer 3 (V.0.4.0, http://frodo.wi.mit.edu/). Additional multiplexing was performed with the *ZFX/ZFY* gene for sexing. The first round of PCR was performed in a 30 μL volume containing 3 μL of neutralization buffer [900 mM Tris-HCl (pH 8.3), 300 mM KCl and 200 mM HCl], 3 μL of K^+-free buffer [30 mM MgCl2, 1.5M Tris-HCl and 1% gelatine], 2.5 mM dNTPs (Takara, Otsu, Shiga, Japan), 0.6 μL of each outer primer (Table 1) and 1 IU Taq DNA polymerase (Takara). The following program was used: 4 min at 96℃; 25 cycles of 30 s at 96℃, 30 s at 62℃, and 1 min at 72℃; and 10 min at 72℃. One micro liter of the first-round PCR products was then reamplified in a second PCR. All PCR reactions contained 1 μL of reaction buffer [20 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween 20, 0.5% Nonidet P-40 and 50% glycerol], 2.5 mM dNTPs, nested primers and 1 IU Taq DNA polymerase, in a total volume of 10 μL. The following program was used: 4 min at 96℃; 28 cycles of 30 s at 96℃, 30 s at 59℃, and 1min at 72℃; and 10 min at 72℃. All reactions were performed in MyCycler Thermal Cycler PCR machines (Bio-RAD, Hercules, CA, USA). Following the nested PCR, 10 μL of the amplification products was electrophoresed on a 2% agarose gel (Cambrex, Rockland, ME, USA) containing ethidium bromide for 40 min at 120 V and then visualized by UV transillumination.

4. Hot start PCR

The methods of conventional multiplex nested PCR were modified from Malcov et al.[8] The first round of PCR was performed in a 25 μL volume containing 3 μL of neutralization buffer [900 mM Tris- HCl (pH 8.3), 300 mM KCl and 200 mM HCl], 3 μL of K^+-free buffer [30 mM MgCl2, 1.5 M Tris-HCl and 1% gelatine], 2 ul of 2.5 mM dNTP (Takara), 0.5 μL of each outer primer (Table 1), 1 μL of dimethyl sulfoxide (Merck) and distilled water. The mixture was preheated to 96℃ for 8 min and was decreased temperature to 75℃. At this time, 5 μL of enzyme mixture was added. The enzyme

Table 1. Primer sets used for preimplantation genetic diagnosis of osteogenesis imperfecta genes

Primer	Mutation	Nucleotide sequence (5'-3')	Annealing Temperature (℃)	Size (bp)
ZFY Fext	*ZFX/ZFY*	ACC(AG)CTGTACTGACTGTGATTACAC	63[b]/72[c]	
ZFY Rext		GCAC(CT)TCTTTGGTATC(CT)GAGAAAGT		
ZFY Fint		A(CT)AACCACCTGGAGAGCCACAAGCT	59	344 bp
ZFY Rint		TGCAGACCTATATTC(AG)CAGTACTGGCA		
Family 1				
US Fext	c.3470 G>A	TGGTGGGCCTCTCCTCCAGAGCTGG	63	
US Rext		TATGGGCATGGGGACCCTGGCATGG		
US Fint		AGCCTTTCTCAAACCATTTC	59	227 bp
US Rint		CACAGAGAGGGAAGAGAGTG		
PT Fext	IVS15+5 G>A	CTCCCCAGGAACTCTAGGGACTGGC	63	
PT Ra		CCTGTGAATGAAATGGAGAT		
PT Fint		GTGGAAATGATGGTGCTACT	59	217 bp
Family 2				
JW Fext	c.3803_3805dup	TCAAACCTTTTCACCAACTT	72	
JW R[a]		TGTAAGTGAACCTGCTGTTG		
JW Fint		TGACCTTGCCTCAGTCTAGT	59	389 bp

Primer Fext/Rext and Fext/R[a] were used in the first round PCR, respectively.
Primer Fint/Rint and Fint/R[a] were used in the second round PCR, respectively.
Annealing temperature of family 1[b] and family 2[c].

mixture consisted of 10 X PCR buffer, 25 mM $MgCl_2$, 1.25 IU Taq polymerase and distilled water. The following program was used: 2 min at 98℃; 10 cycles of 1 min at 96℃, 2 min at 62℃ and 3 min at 72℃; 6 cycles of 45 s at 94℃, 1 min at 62℃ and 3 min at 72℃; and 8 min at 72℃. The second round of PCR was performed in a 10 μL volume containing 1 μL of reaction buffer [20 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween 20, 0.5% Nonidet P-40, 50% glycerol], 1 μL of 2.5 mM dNTPs, 0.75 μL of each inner primer, 1 μL of dimethyl sulfoxide and distilled water. The following program was used: 8 min at 96℃ and 30 s at 75℃ (for addition of the enzyme mixture); 14 cycles of 2 min at 98℃, 1 min at 96℃, 2 min at 60℃ and 2 min at 72℃; 20 cycles of 45 s at 94℃, 1 min at 60℃ and 2 min at 72℃; and 10 min at 72℃. The second enzyme mixture consisted of 10 X PCR buffer, 25 mM $MgCl_2$, 2 IU Taq polymerase and distilled water. All reactions were performed in MyCycler Thermal Cycler PCR machines (Bio-RAD). Following the nested PCR, 10 μL of amplification products was electrophoresed on a 2% agarose gel (Cambrex) containing ethidium bromide for 40min at 120 V and then visualized by UV transillumination.

5. Restriction fragment length polymorphism (RFLP) analysis

RFLP analysis was performed by mixing 10 μL of the *ZFX/ZFY* second-round PCR products with10 IU of the restriction enzyme HaeIII (NEB, Ipswich, MA, USA) and 2 μL of the supplied buffer in a total volume of 20 μL. The reaction tubes were incubated 37℃ for 4 h. The products of the enzymatic restriction digest were resolved by electrophoresis on a 4% Nusieve agarose gel (Cambrex) containing ethidium bromide for 50 min at 120 V and then visualized by UV transillumination.

6. Embryo biopsy

The embryo biopsy was performed on the third day at the six to eight-cells stage. The biopsy and dissection of the zona pellucida were performed using a micromanipulator (Narshige, Tokyo, Japan) mounted on an inverted microscope (Nikon, Tokyo, Japan). The presence of a clearly visible nucleus guided the selection of the blastomere to be biopsied in G-PGD medium (Virolife, Goteborg, Sweden). Also, we decided on a number of biopsied blastomeres according to the cell stage. Biopsied one (embryo ≤ 6 blastomere) or two blastomeres (embryos ≥8 blastomere) were loaded in 3 μL of ALB buffer, and the genetic analysis was performed on the same day. Additionally, we tested removing some of the final wash drop as a negative control.

7. Ethics

The present study was approved by the Institutional Review Boards of Seoul National University Hospital (# 0909-056-296).

8. Statistical analysis

The amplification rate and the rate of allele dropout (ADO) were compared using t-test. A value of $P<0.05$ was considered statistically significant.

Results

1. Amplification rate

The PCR conditions were set up separately for each locus, and amplification success was judged by the presence of a single specific and correctly sized band of reasonable intensity for secondary PCR products analyzed on agarose gels. We obtained products of the secondary PCR corresponding to the mutation in each case.

The amplification rates for hot start PCR and conventional multiplex nested PCR were evaluated using single lymphocytes isolated from two couples. At least 50 cells from each mutation were analyzed for each method. The amplification efficiency for the first couple (family 1) ranged from 94.0% to 96.0% with hot start PCR and from 86.0% to 98.0% with conventional multiplex nested PCR (Table 2). The total amplification rate for hot start PCR was higher than for the conventional method but was not significantly higher. The results for the second couple (family 2) ranged from 89.0% to 95.0% with the hot start PCR method and from 69.0% to 91.0% with the multiplex nested PCR method. The results for family 2 were statistically significant ($P<0.001$).

Figure 1 shows the PCR bands obtained for each procedure of c.3470 G>A in *COL1A1* from family 1. We were confirmed from these results that hot start PCR lessened nonspecific products.

2. ADO rate

OI is an autosomal dominant disorder, but additional multiplexing was performed with the *ZFX/ZFY* gene for sexing, for which we calculated the ADO rate. The rate of ADO was determined by HaeIII digestion of the *ZFX/ZFY* nested PCR products. Second-round PCR products from XX cells (344 bp) were cut into two fragments of 300 bp and 44 bp, and XY cells had four fragments of 300, 216, 84 and 44 bp. Analyzing single lymphocytes from the male individuals that gave positive amplification signals provided information on the degree of ADO at *ZFX/ZFY*. The data shown in Table 2 reveal that in family 1, the rates of ADO, 42.0% and 36.0%, were not statistically different. However, in family 2, a statistically lower rate of ADO was obtained with the hot start PCR procedure (6.0%) than with the other method.

3. Clinical case of PGD

Following establishment of the hot start PCR protocol, PGD was performed in the clinic for family 2. The couple visited the IVF clinic for PGD in order to have an unaffected baby. The husband was normal; the wife was diagnosed with OI due to a duplication of c.3803_3805 in *COL1A2*. They underwent a single IVF-PGD treatment cycle, although the female partner had a poor reaction of the ovarian stimulation. The results from this case are sum-

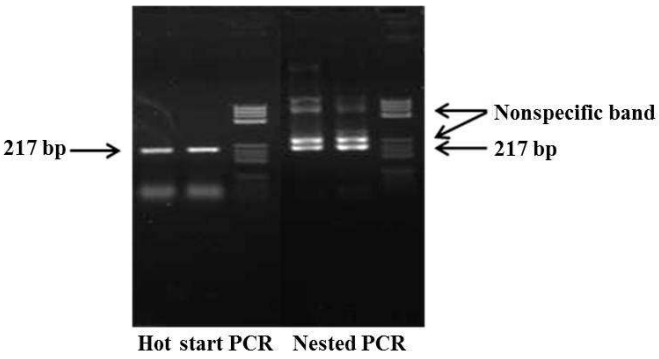

Fig. 1. Results of single lymphocyte genetic analysis using hot start PCR (left) and conventional multiplex nested PCR (right). Products from the second round of PCR for c.3470 G>A in *COL1A1* from family 1 are shown.

Table 2. Comparison of PCR amplification efficiency and allele dropout using hot start PCR and multiplex nested PCR

	Mutation	PCR amplification efficiency			Allele dropout		
		Hot start PCR	Multiplex nested PCR	*P*-value	Hot start PCR	Multiplex nested PCR	*P*-value
Family 1	c.3470 G>A in *COL1A1*	48/50 (96.0)	49/50 (98.0)	0.284	21/50 (42.0)	18/50 (36.0)	0.355
	IVS15+5 G>A in *COL1A1*	47/50 (94.0)	43/50 (86.0)	0.080			
	ZFX/ZFY	96/100 (96.0)	95/100 (95.0)	0.354			
	total	191/200 (95.5)	187/200 (93.5)	0.174			
Family 2	c.3803_3805 dup in *COL1A2*	95/100 (95.0)	91/100 (91.0)	0.104	3/50 (6.0)	7/50 (14.0)	<0.05
	ZFX/ZFY	89/100 (89.0)	69/100(69.0)	<0.001			
	total	184/200 (92.0)	160/200 (80.0)	<0.001			

The values are expressed as numbers (%). dup, duplication

Table 3. Clinical results from the preimplantation genetic diagnosis for osteogenesis imperfecta in family 2

Cell No.		Cell stage	Amplification efficiency (+, −)			Sex	ADO	Result
			COL1A2	*ZFX/ZFY*	Positive control			
1	1-1	8C	+	+	-	M	-	A
	1-2		+	+	-	M	-	A
2	2-1	8C	+	+	-	M	-	A
	2-2		+	+	-	M	-	A
3	3-1	8C	+	+	-	F	-	NA
	3-2		+	+	-	F	-	NA

A, affect; ADO, allele dropout; M, Male; F, female; NA, non-affective.

marized in Table 3.

For family 2, five oocytes were collected, three oocytes were suitable for intracytoplasmic sperm injection (ICSI), and three oocytes were fertilized normally. Two blastomeres were biopsied from each embryo on day 3 and were analyzed with positive and negative controls (Table 3). In this case, it was not failed amplification and ADO. We selected an unaffected embryo after DNA purification and direct sequencing of the secondary PCR products from the hot start PCR method. Only one unaffected embryo was available for transfer to the uterus on day 5 after the PGD analysis. However, pregnancy was not achieved.

Discussion

The purpose of this study was to compare the efficacy of the hot start PCR with that of conventional multiplex nested PCR. Our experiments showed that the rate of amplification for hot start PCR is higher than for conventional multiplex nested PCR. And there were fewer nonspecific bands and the PCR band appeared clearly at the correct size.

The results of ADO in family 1 were 42.0% and 36.0% unlike family 2. The most experienced PGD laboratories are generally able to reduce ADO rates to 5.0-15.0%,[10] but ADO occurred on average in 25.8% of the samples, ranging between 0.0% and 60.0%.[11,12] ADO may be caused by a combination of inefficient denaturation and degradation of one of the genomic alleles in the first cycles of PCR.[13] We were conjectured the result from the long period of storage (>12 month) of lymphocyte in the refrigerator. There was a problem of the donated blood sampling once again.

PCR is an elaborate technique for amplifying small amounts of target DNA, but clinical PGD-PCR cycle requires particular care. PGD for monogenic diseases is susceptible to problems of contamination, PCR failure, and most significantly, ADO, which may lead to misdiagnosis.[14,15] We take about three to six months to set up a PCR condition, and it was tested for DNA dilution, single lymphocyte and some blastomeres. It was mostly difficult in that time of diverting from a single level of DNA to a single cell. Therefore, most researchers in a PGD clinic spend much time determining the PCR condition for each PGD cycle.

Taq polymerase is thermostable DNA polymerase that is named after the thermophilic bacterium from which it was originally isolated by Thomas D. Brock in 1965.[5] Although the Taq polymerase grows optimally at 70℃ and cannot grow at moderate temperatures, its DNA polymerase has significant activity at 20 -37℃. This activity is a bane to some PCRs, because it catalyzes non-specific priming.[16] With hot start amplification, both primer/template mispriming and the formation of primer dimers, the main sources of undesirable nonspecific DNA amplification, are largely prevented, leading to significantly improved sensitivity and specificity.[17,18]

Hot start PCR methods can be classified into four large groups: methods that use physical removal of essential reaction components, those that use sequestration of components within the reaction, those that use chemical modification of the polymerase and those that use reversible, ligand-mediated polymerase inhibition. We chose a method that uses the physical removal of essential reaction components. The physical removal method does not require additional materials and reduces the expenses for hot start PCR. Many researchers and companies have improvement to increase the convenience of hot start PCR. For instance, Taq Start antibody provides automatic hot start for increased enzyme specificity and product yield and, cold -sensitive mutations in Klentaq markedly reduce the enzyme's activity at 37℃ yet allow it to have apparently normal activity at 68℃ and resistance at 95℃.[19,20]

Malcov et al. applied the hot start PCR method for PGD for Duchenne muscular dystrophy.[21] However, this report did not compare conventional multiplex nested PCR with hot start PCR. Amplification-based tests can be used for the diagnosis of monogenic defects at the DNA level of a single cell.[22,23] Thus, not only methods of DNA amplifi-

cation and DNA extraction, but also guideline for PGD have been examined. The accurate amplification of a specific region is the most important criterion in the clinical use of PGD.[24-26] In conclusion, it is suggested that the hot start PCR method may be a useful method for PGD-PCR that could improve the success of diagnosis. But this data have obtained only two families. Much more research remains to be done on diverse PGD cases for the method could have the statistical significance.

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