

Rapid Prenatal Detection of Down and Edwards Syndromes by Fluorescent Polymerase Chain Reaction with Short Tandem Repeat Markers

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Chromosome analysis is the main tool for the prenatal diagnosis of trisomies but requires great technical expertise and time consuming manual procedures. Recently, alternative methods, which provide rapidity and accuracy, without culture, have been developed for pregnant women requiring rapid decisions for termination. In this study, multiplex fluorescent polymerase chain reactions (F-PCRs) were performed by the concurrent use of short tandem repeat (STR) markers specific for the chromosomes 18 and 21. The aims of this investigation were to evaluate the clinical usefulness of this assay for rapid prenatal detection of Down and Edwards syndromes and then to accumulate the basic data for clinical application. F-PCRs were carried out using DNA extracted from amniotic fluid and peripheral blood derived from 47 normal karyotypes, 23 Down and 8 Edwards syndrome patients. Fluorescent intensities of the PCR products were then calculated. Normal samples displayed diallelic peaks for each STR marker. Reference ranges of peak area ratios were 1.0-1.4 for D21S11, 1.0-1.5 for D21S1412 and 1.0-1.3 for D18S535 and D18S51. Down and Edwards syndromes showed characteristic triallelic peaks of similar intensity corresponding to 3 different alleles or characteristic diallelic peaks. The sensitivity, specificity and efficiency of the assay for detecting Down and Edwards syndromes were 96.7%, 93.6% and 94.8%, respectively.

In conclusion, these results show that F-PCR rapidly detects Down and Edwards syndromes with high accuracy and provides normal reference ranges of peak area ratios. However, the presence of false results (4 out of 77 cases) and the possibility of anomalies other than trisomies 21 and 18 do

not permit F-PCR to substitute for chromosome analysis.

Key Words: Fluorescent polymerase chain reaction, Down syndrome, Edwards syndrome, Prenatal detection, Short tandem repeats, D18S535, D18S51, D21S11, D21S1412

INTRODUCTION

Trisomies cause 17% of fetal deaths.¹ Moreover, the great majority of trisomies in newborns involve chromosomes 21, 18, and 13² and almost all of these are trisomies 21 (Down syndrome) or 18 (Edwards syndrome). For the prenatal diagnosis of chromosomal abnormalities, cytogenetic analysis is routinely performed using *in vitro* cultures of fetal cells derived from amniotic fluid, chorionic villi or fetal blood. However, *in vitro* culture requires sufficient cells, great technical expertise and time consuming manual procedures, and occasionally may result in pseudomosaicism. Its main disadvantage is that an interval of 2 weeks between sampling and definitive result is required. Particularly, when ultrasound or biochemical analyses already suggest a fetal chromosomal disorder and a decision has not been made at 20 weeks gestation, the anxiety of waiting for the result and the time delay before therapeutic interventions can be instituted create difficulties for pregnant women and clinicians. Recently, alternative methods, which are rapid and accurate and do not require culture, have been developed for pregnant women requiring a rapid decision upon termination. As alternatives, interphase fluorescent *in*

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situ hybridization (FISH) using a single probe or multiple color probes on uncultured amniotic fluid and quantitative fluorescent polymerase chain reaction (QF-PCR) assays have been devised. Even though FISH using commercialized probes has been reported to be a highly specific and sensitive diagnostic technique,^{3,4} uncultured amniotic fluid decreases the effectiveness of interphase FISH due to the nonspecific binding of fluorescent probes to the cytoskeleton and the unclear background,⁵ and the results are available after 48 hours. Recently, it has been shown that the rapid and accurate detection of aneuploidies can be obtained by QF-PCR assays using highly polymorphic short tandem repeat (STR) markers. Mansfield et al. developed a chromosome dosage strategy using QF-PCR, which exploits the existence of particular groups of STR sequences in the human genome⁶ and since then efforts have continued to develop clinical applications.⁷⁻¹¹

In the present study, two separate multiplex fluorescent polymerase chain reactions (F-PCRs) were performed by the concurrent use of STR markers specific for each of chromosomes 18 and 21, to detect Down and Edwards syndromes simultaneously. The aims of current investigation were to evaluate the clinical usefulness of this assay for the rapid prenatal detection of Down and Edwards syndromes, and then to establish reference ranges for clinical application.

MATERIALS AND METHODS

Subjects

During the period from July, 1999 to February, 2000, 39 amniotic fluid and 39 peripheral blood samples were referred to cytogenetic laboratory in Seoul Clinical Laboratories for cytogenetic analysis were collected with both wet material and archived slides (Table 1). Indications for cytogenetic analysis included: positive maternal serum triple test for Down and Edwards syndrome, advanced maternal age, ultrasound anomalies, a family history of chromosomal abnormality or dysmorphism. The karyotypes of all amniotic fluid and peripheral blood were assessed by conventional cytogenetic analysis.

Fluorescent PCR

Genomic deoxyribonucleic acid (DNA) was extracted from 1 to 3 mL of amniotic fluid or 500 μ L of peripheral blood using a standard phenol-chloroform extraction procedure.¹² For archived slides, DNA was extracted with the procedure previously described.¹³

To amplify the 4 STRs specific for chromosomes 18 and 21, we used the primer sets synthesized by PE Biosystems (Foster City, California, USA), which were produced to request and are listed in Table 2. The first primer set, D18S535 and

Table 1. Distribution of Specimen Types and Karyotypes in 78 Subjects

	Normal	Trisomy 21			Trisomy 18		Total
		+21	T(21q;Dq/Gq)*	Mosaic	+18	18q+ [†]	
Amniotic fluid							
Wet material	21	11	0	0	3	0	35
Archived slides	0	0	1	1	2	0	4
Peripheral Blood							
Wet material	26	9	1	0	1	0	37
Archived slides	0	0	0	0	1	1	2
Total	47	20	2	1	7	1	78

*The net imbalance is trisomy for the long arm of chromosome 21 and loss of the short arm of a D or G group chromosome by Robertsonian translocation between 21q and Dq or Gq.

[†]Partial trisomy of 18q results from partial duplication of 18q or unbalanced translocation of 18q.

Table 2. Primers Specific for Each Polymorphic Short Tandem Repeat used in Fluorescent Polymerase Chain Reaction

Markers	Sequence of primers	Chromosome location	Heterozygosity	Size of PCR products(bp)	Label
D18S535(F) D18S535(R)	5'-TCA-TGT-GAC-AAA-AGC-CAC-AC-3' 5'-AGA-CAG-AAA-TAT-AGA-TGA-GAA-TGC-A-3'	18q12.2 - 12.3	0.92	≥138	5'-TET*
D18S51(F) D18S51(R)	5'-CAA-ACC-CGA-CTA-CCA-GCA-AC-3' 5'-GAG-CCA-TGT-TCA-TGC-CAC-TG-3'	18q21.3	0.87	271 - 343	5'-TET
D21S11(F) D21S11(R)	5'-TAT-GTG-AGT-CAA-TTC-CCC-AAG-TGA-3' 5'-GTT-GTA-TTA-GTC-AAT-GTT-CTC-CAG-3'	21q21	0.93	172 - 264	5'-FAM [†]
D21S1412(F) D21S1412(R)	5'-CGG-AGG-TTG-CAC-TGA-GTT-G-3' 5'-GGG-AAG-GCT-ATG-GAG-GAG-A-3'	21q22.3	0.80	≥305	5'-FAM

*TET: Tetrachloro-6-carboxyfluorescein.

[†]FAM: 6-Carboxyfluorescein.

D21S11¹⁴ and the second primer set, D18S51¹⁵ and D21S1412 were used in two separate multiplex F-PCRs. The forward oligonucleotide primers were 5' end labeled with 6-carboxyfluorescein (5'-FAM, blue) or tetrachloro-6-carboxyfluorescein (5'-TET, green) and different fluorochromes were used to identify differently sized PCR products.

PCR amplification was performed in a total volume of 20 μ L containing 50-200 ng of genomic DNA, GeneAmp 1 \times PCR buffer II (PE Biosystems, Foster City, California, USA), 2 mM MgCl₂, 200 μ M deoxynucleoside triphosphates mix (Promega Madison, WI, Sweden), 1 U of AmpliTaq Gold DNA polymerase (PE Biosystems, Foster City, California, USA), 1.5 to 5 pmol of each primer. After hot-start PCR at 94°C for 10 minutes, denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds and extension at 72°C for 45 seconds were carried out for 33 cycles.⁸

Electrophoretic analysis of the fluorescent PCR products

Two μ L of each PCR product for D21S11 [FAM]/D18S535[TET] and D21S1412[FAM]/D18S51[TET] were mixed with 16 μ L of deionized water. One μ L of the reaction mixture was then mixed with loading buffer containing 12 μ L of deionized formamide and 0.5 μ L of GS-500 TAMRA (PE Biosystems, Foster City, California, USA) containing the internal size standard. The mixture was then denatured at 95°C for 5 minutes

and chilled on ice. Capillary electrophoretic analysis was performed using POP4 gel (PE Biosystems, Foster City, California, USA) and an ABI PRISM 310 genetic analyzer (PE Biosystems, Foster City, California, USA). The relative fluorescence intensities of the PCR products were calculated with GeneScan analysis 2.1 software (PE Biosystems, Foster City, California, USA).

Analysis of STR markers for polymorphisms

The polymorphisms for each STR marker specific for chromosome 21 (D21S11, D21S1412) and for chromosome 18 (D18S535, D18S51) were analyzed for each of the normal karyotype, Down and Edwards syndrome. The Wilcoxon rank sum test was used to compare the median value of the peak area ratios of the normal karyotype samples and the Down or Edwards syndrome samples. *p* values of less than 0.01 were considered significant. In cases of heterozygous samples showing diallelic peaks, the normal reference ranges of peak area ratios were calculated to differentiate between patterns of Down or Edwards syndrome from the normal karyotype patterns.

Evaluation of STR markers for clinical usefulness

The frequencies of disomy 21 or trisomy 21 in samples showing homozygous, and thus the uninformative pattern were calculated for STR

markers specific for chromosome 21. Frequencies of disomy 18 or trisomy 18 samples showing the uninformative pattern were calculated for STR markers specific for chromosome 18.

Evaluation of F-PCR for clinical usefulness

The negativity of F-PCR was determined as being diallele with the peak area ratio within normal reference range for all the informative STR markers, the positivity of F-PCR was determined as being triallele or diallele with the peak area ratio above normal reference range for all the informative STR markers. False positive, false negative, true positive and true negative by F-PCR were defined by comparing the results of F-PCR with confirmative cytogenetic results. The performance of F-PCR for detecting Down and

Edwards syndromes was then calculated.

RESULTS

Analysis of STR markers for polymorphism

Normal karyotype samples displayed diallelic peaks for each STR marker (Fig. 1). The reference ranges of the peak area ratios were 1.0-1.4 for D21S11, 1.0-1.5 for D21S1412 and 1.0-1.3 for D18S535 and D18S51 (Table 3). Down (Fig. 2) and Edwards syndromes (Fig. 3) showed characteristic triallelic peaks of similar intensity, which corresponded to the 3 different alleles or characteristic diallelic peaks. In Down syndrome, peak area ratios were 1.5-3.0 for D21S11 and 1.8-3.1 for D21S1412 (Table 4), in Edwards syndrome,

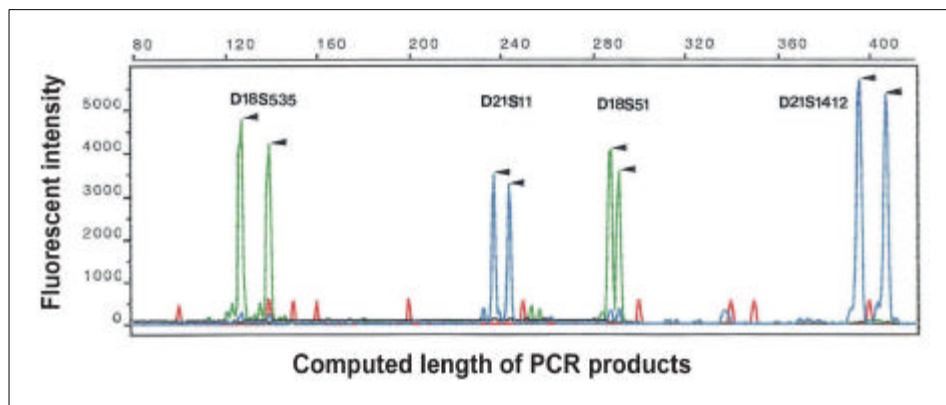


Fig. 1. Electrophoretogram of fluorescent PCR amplified STRs of a normal karyotype sample showing a diallelic pattern with fluorescent intensity ratios of 1.0 - 1.5 for D18S 535, D21S11, D18S51 and D21S1412 STR markers. The X-axis shows the computed lengths of the PCR products in base pairs as determined automatically using the internal size standard, GS-500 TAMRA. The Y- axis shows fluorescent intensities in arbitrary units.

Table 3. Polymorphism of Normal Karyotype Samples (n=47) for Each STR Marker Specific for Chromosome 18 (D18S535 and D18S51) or 21 (D21S11 and D21S1412)

Marker	Heterozygous samples (n)	Homozygous samples (n)	Ratio of peak areas * in heterozygous samples		
			Range	Mean	2.5-97.5 percentile [†]
D18S535	42	5	1.0 - 1.4	1.1	1.0 - 1.3
D18S51	42	5	1.0 - 1.5	1.1	1.0 - 1.3
D21S11	39	8	1.0 - 1.5	1.2	1.0 - 1.4
D21S1412	43	4	1.0 - 1.5	1.2	1.0 - 1.5

* Peak area ratios were calculated by dividing the larger peak area by smaller area for the relative quantification of PCR products.

[†]The reference range of the peak area ratio was defined as the peak area ratio within the 2.5-97.5 percentile range in the normal karyotype group. Three samples showing false positives were excluded from the reference range calculation.

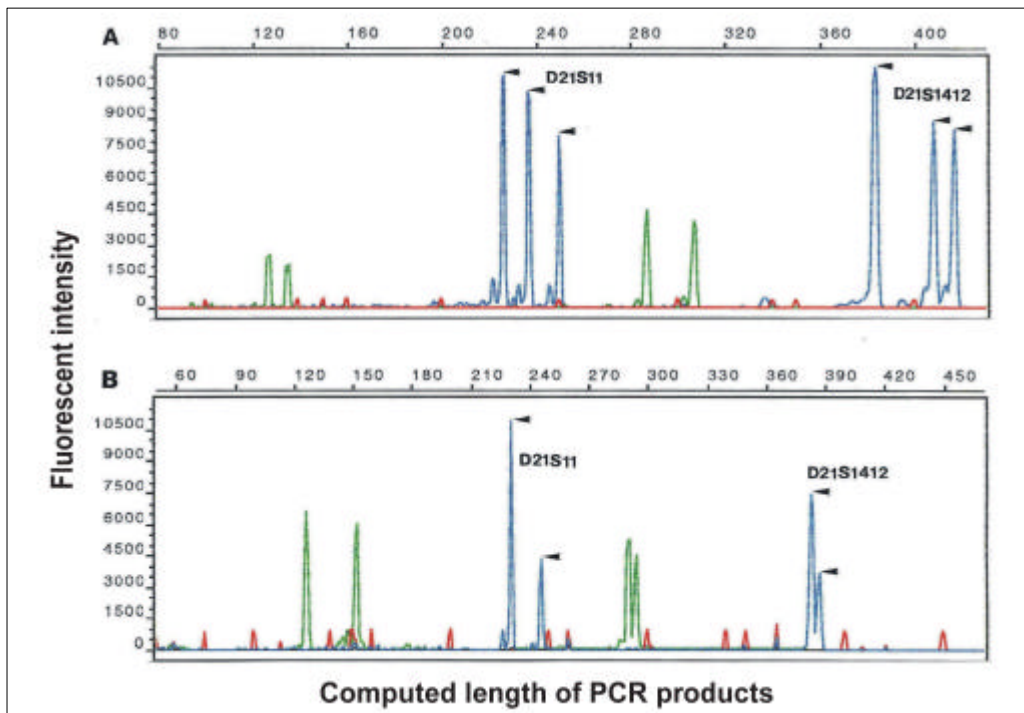


Fig. 2. Electrophoretograms of fluorescent PCR amplified STRs of trisomy 21 samples demonstrating a characteristic (A) triallelic pattern with 3 STR peaks corresponding to 3 different alleles and (B) a diallelic pattern with a fluorescent intensity ratio of about 2.0 for D21S11 and D21S1412 STR markers.

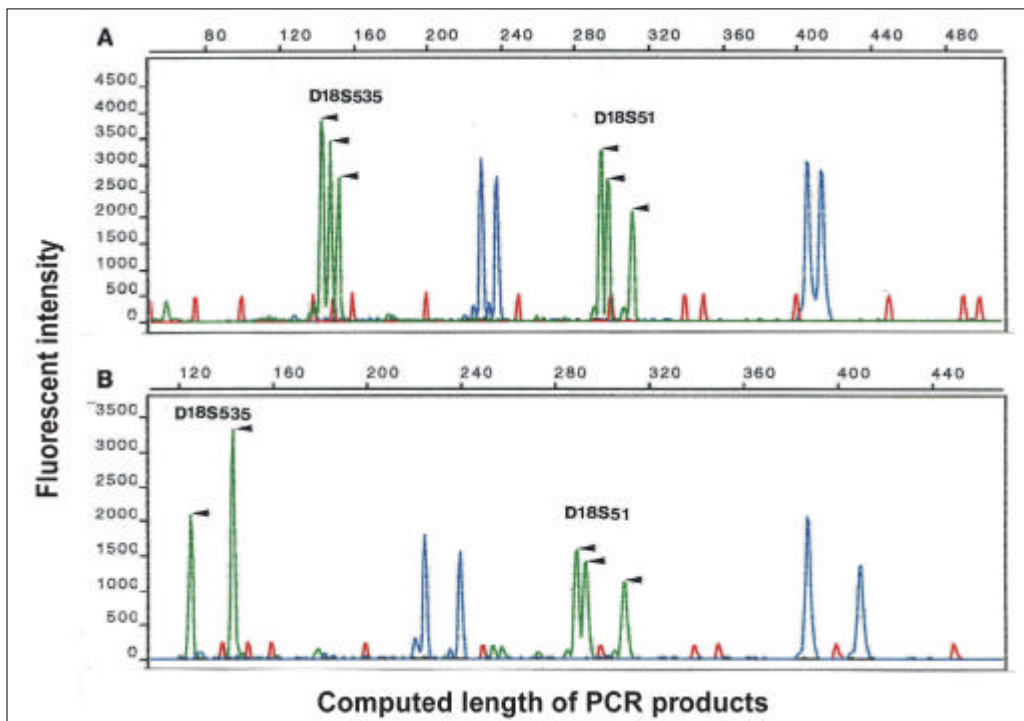


Fig. 3. Electrophoretograms of fluorescent PCR amplified STRs of trisomy 18 samples demonstrating a characteristic (A) triallelic pattern with 3 STR peaks corresponding to 3 different alleles for D18S535 and D18S51 STR markers and (B) a diallelic pattern with a fluorescent intensity ratio of about 2.0 for D18S535 marker and a triallelic pattern for D18S51 marker.

peak area ratios were 1.6-1.9 for D18S535 and 1.6-3.4 for D18S51 (Table 4). However, 3 samples showing false positive (Table 3), 1 sample showing false negative and 1 sample of partial trisomy of 18q (Table 4) were excluded from calculating the above range values because they were calculated for the purpose of clinical application (Table 5). A significant difference was found in the peak area ratios of the normal karyotype samples and the Down ($p < 0.01$) or the Edwards syndrome samples ($p < 0.01$) (Fig. 4).

F-PCR using amniotic fluid allowed the simultaneous prenatal diagnosis of Down and Edwards syndromes within 8 hours. In particular, 2 cases of Down syndrome with a Robertsonian translocation and 1 case of partial duplication of 18q were correctly diagnosed.

Evaluation of STR markers for clinical usefulness

The concurrent use of 2 STR markers specific

Table 4. Polymorphism of Trisomy 21 Samples (n=23) and Trisomy 18 Samples (N=8) for Each STR Marker Specific for Chromosome 18 (D18S535 and D18S51) or 21 (D21S11 and D21S1412)

Marker	Triallelic samples (n)	Diallelic samples (n)	Monoallelic samples (n)	Ratio of peak areas* in diallelic samples		
				Range	Mean	SD
Trisomy 21 samples						
D18S535	0	22	1	1.0 - 1.5	1.2	0.15
D18S51	0	17	6	1.1 - 1.5	1.2	0.11
D21S11	4	17	2	1.5 - 3.0	2.2	0.42
D21S1412	10	8	5	1.8 - 3.1	2.3	0.48
Trisomy 18 samples						
D18S535	3	5	0	1.6 - 1.9	1.7	0.14
D18S51	2	6	0	1.6 - 3.4	2.1	0.67
D21S11	0	8	0	1.1 - 1.4	1.2	0.10
D21S1412	0	5	3	1.2 - 1.5	1.3	0.14

*Peak area ratios were calculated by dividing the larger peak area by the smaller area in diallelic samples. One sample showing a false negative among the trisomy 21 samples and a sample of partial trisomy of 18q among trisomy 18 samples were excluded from the peak area ratio calculation.

Table 5. Summary of Fluorescent PCR Results in Normal Karyotype, Down and Edwards Syndrome Samples

	Normal	Trisomy 21			Trisomy 18		Total
		+21	Der(21q;Dq or Gq)	Mosaic	+18	18q+	
True positive	0	19	2	0	7	1	29
False positive	3	0	0	0	0	0	3
True negative	44	0	0	0	0	0	44
False negative	0	0	0	1	0	0	1
Total	47	19	2	1	7	1	77*

*An uninformative sample was excluded from the total number of subjects.

†Fluorescent PCR results were confirmed by cytogenetic analysis.

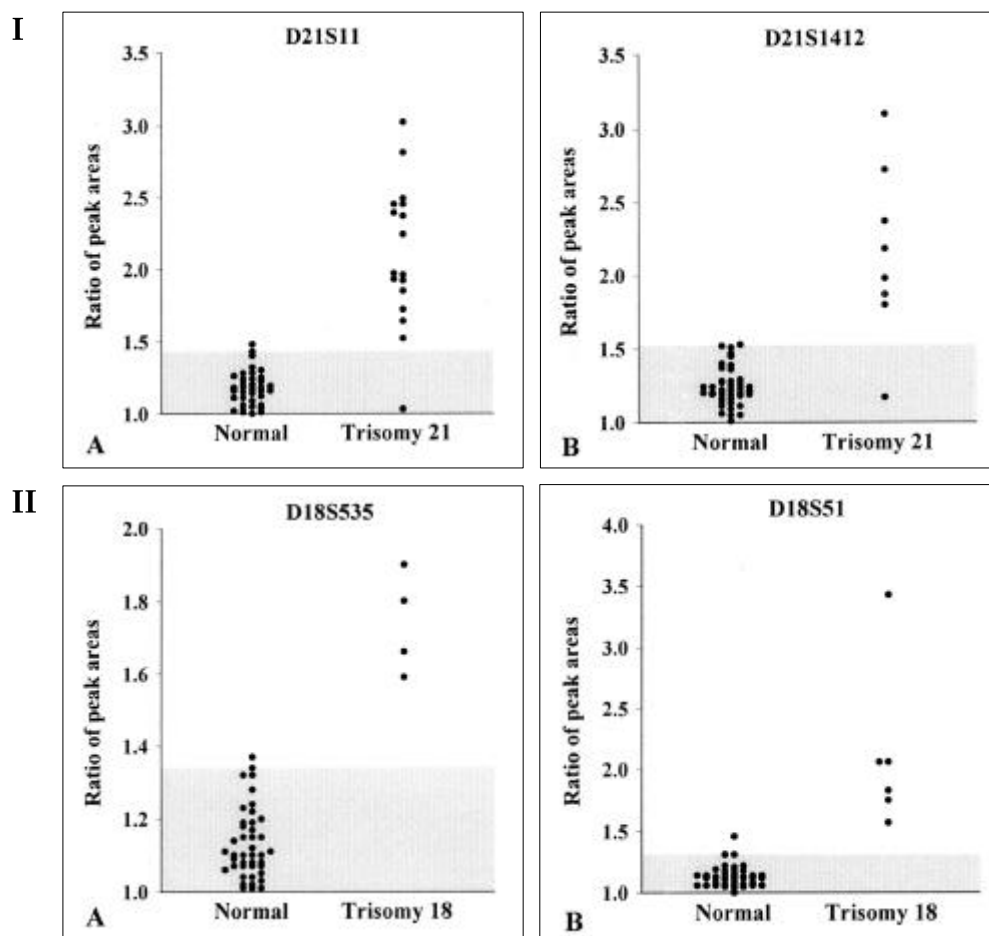


Fig. 4. I. Distribution of the peak area ratios of D21S11 (A) and D21S1412 (B) STRs in normal heterozygotes and diallelic trisomy 21. II. Distribution of the peak area ratios for D18S535 (A) and D18S51 (B) STRs in normal heterozygotes and in diallelic trisomy 18, excluding a sample of partial trisomy of 18q. Shaded areas indicate the normal reference ranges (up to 97.5 percentile of normal heterozygotes).

for each of the chromosomes 18 and 21 markedly decreased the frequencies of uninformative samples. All the other samples except for 1 sample of Down syndrome were heterozygous and informative for at least one marker specific for each of the analyzed chromosomes (Table 6).

Evaluation of F-PCR for clinical usefulness

The diagnostic sensitivity, specificity and efficiency of the assay for the detection of Down and Edwards syndromes were 96.7%, 93.6% and 94.8%, respectively. The predictive value of the positive test, the predictive value of the negative test, the false positive rate and the false negative rate were 90.6%, 97.8%, 6.4% and 3.3%, respec-

tively. However, one uninformative sample (Table 6) was excluded from calculating the above values.

DISCUSSION

On the assumption that, within the exponential phase of PCR amplification, the amount of PCR product is proportional to quantity of the initial target sequence, and therefore, to the numbers of the chromosome investigated,¹⁶ quantitative PCR methods have been devised to detect aneuploidies. Modified quantitative PCR using highly polymorphic STRs specific for each chromosome has been successfully applied to detect trisomies

Table 6. Frequencies of Samples Showing Homozygous and thus the Uninformative Pattern for Each STR Marker and Combined STR Markers in Disomy 21 (n=55), Disomy 18 (n=70), Trisomy 21 (n=23) and Trisomy 18 (n=8)

STR marker	Disomy 21	Disomy 18	Trisomy 21	Trisomy 18
	homozygous	homozygous	monoallelic	monoallelic
	n (%)	n (%)	n (%)	n (%)
Chromosome 18 marker				
D18S535		6 (8.6%)		0 (0.0%)
D18S51		11 (15.7%)		0 (0.0%)
D18S535 + D18S51		0 (0.0%)		0 (0.0%)
Chromosome 21 marker				
D21S11	8 (14.5%)		2 (8.7%)	
D21S1412	7 (12.7%)		5 (21.7%)	
D21S11 + D21S1412	0 (0.0%)		1 (4.6%)	

21 and 18.^{6,8,9} STRs are variable in terms of the number of tandemly repeated units from one allele to another with repeat elements of 2 to 4 bp in length.¹⁷ Therefore, STRs have been widely used as polymorphic markers in human genome. Thus, the great majority of normal samples are heterozygous for each of these markers, and consequently, quantitative PCR products show diallelic peaks of similar fluorescence intensities. Moreover, a peak area ratio of 1 : 1 corresponds to two alleles at each STR locus. Only a minority of normal samples from homozygous individuals show a single STR peak.¹⁸ Trisomic samples are expected to fall into two major groups; one group presenting a "triallelic" pattern with three STR peaks of similar intensity and ratios of 1 : 1 : 1 corresponding to three different STR alleles, and another group displaying a "diallelic" pattern of two peaks with a ratio of 2 : 1, corresponding to two identical STR alleles and a third allele. In order to increase the accuracy of this assay, we selected primers known to be highly heterozygous in the human population¹⁰ and chose to amplify STR within the chromosome region (e.g., 21q22.2-21q22.3, 18q12.3-18q22.1), which are known to cause the severe clinical features of trisomies (Table 2).^{19,20} As a result, a case of trisomy 21 by Robertsonian translocation and a case of partial duplication of 18q were diagnosed. Particularly,

the sample of dup(18)(q21q23) correctly showed diallelic peaks with a ratio of 1 : 1 for D18S535 STR marker and a ratio of 1 : 1.8 for D18S51 STR marker. Differential labeling and the concurrent use of fluorescent primers specific for each chromosome allowed the simultaneous detection of the most frequent autosomal aneuploidies, trisomies 21 and 18. As the result of using two markers specific for each of the analyzed chromosomes, only one of 78 samples showed an uninformative pattern.

One case of low level mosaicism for trisomy 21 with a karyotype of 46,XX,+21,der(21;21)(q10;q10)[10]/46,XX[120] produced a false negative result (Table. 5). However, Pertl et al. were able to diagnose mosaic cases for trisomy, although not enough cases have been investigated to establish the level of sensitivity of F-PCR tests in samples with low levels of mosaicism. Three normal karyotype samples produced false positive results in the current study. The most likely explanation is that these resulted from small amounts of DNA extracted from the archived slides or heparinized blood. In Sherlock's study, artificial ratios were never seen when 10 or more cells were analyzed, probably because any single cell within the group that undergoes preferential amplification of only one allele is counterbalanced by the normal amplification in the majority of cells, and by the

preferential amplification of the other allelic sequence in a few single cells.¹¹ Optimizing the concentrations of the different primers to equalize the efficiency of annealing is a critical point for the success of multiplex F-PCRs, as was observed from the preliminary experiments performed in this study.

An F-PCR test using several samples can be performed within 8 to 24 hours depending on how the demands of the situation. The major advantages of this multiplex F-PCRs are to reduce the time and the number of cells required for prenatal diagnosis and to avoid the difficulties in differentiating pseudomosaicism, caused by *in vitro* culture by true mosaicism. F-PCR assays can also be used to analyze small quantities of genetic material, such as DNA of fetal nucleated erythrocytes obtained from maternal blood.²¹ The simultaneous analysis of the STR profile from maternal and paternal blood could enforce the diagnostic value of this assay by excluding the possibility of maternal cell contamination and of a false result, and by determining the maternal or paternal origin of the nondisjunction.²²

In conclusion, these results show that F-PCR rapidly and concurrently detects Down and Edwards syndromes with high accuracy and provide normal reference ranges for clinical application. If F-PCR is extended to parental studies using peripheral blood, this assay could be useful for the confirmative diagnosis of Down and Edwards syndromes. However, the presence of false results (4 out of 77 cases) and the possibility of chromosomal abnormalities other than trisomies 21 and 18 do not permit F-PCR to substitute for chromosome analysis.

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