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Note

Quantitative Fluorescent PCR – A Rapid Approach to Prenatal Diagnostics of Common Autosomal Aneuploidies

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Autosomal trisomies account for more than 80 % of significant chromosomal disorders and are routinely detected by the cytogenetic analysis of cultivated amniotic fluid cells. However, this approach is time-consuming and requires a significant level of training and expertise. The main aim of our work was to introduce QF-PCR to our lab, a quicker, simpler and cheaper method. We also aimed to evaluate the usefulness of the chosen marker set in the Croatian population and the reliability and accuracy of the obtained results. STR loci from chromosomes 13, 18 and 21 were co-amplified, separated by capillary electrophoresis and analysed. Characteristic triplets and/or 2:1 patterns were detected for trisomic samples while normal samples were either homozygous or heterozygous. The tested set of loci showed high heterozygosity and therefore a good potential for analyzing the Croatian population. The results of QF-PCR were in full compliance with the cytogenetic analysis which was also performed for cultivated amniotic fluid cell samples.

Keywords
QF-PCR
prenatal diagnostics
chromosomal aneuploidy
amniotic fluid

INTRODUCTION

Autosomal trisomies account for more than 80 % of significant chromosomal disorders and are routinely detected by the cytogenetic analysis of cultivated amniotic fluid cells. However, this approach is time-consuming and requires a significant level of training and expertise. Therefore, need for a quicker method emerged which would significantly decrease both the anxiety period for the parents and the financial input required for the analysis. Single or multiple colour fluorescence *in situ* hybridization (FISH) on uncultured amniotic fluid cells was introduced first,[1] offering high sensitivity and significantly shorter time required to obtain the results. Shortly afterwards, a new kind of assay was designed, combining the properties of PCR and fluorescent labelling.[2] Quantitative fluorescent polymerase chain reaction (QF-PCR) detects the copy number of target chromosomes by amplifying microsatellite (STR) sequences on polymorphic loci. Its advantages over FISH reflect in the fact that less starting material is needed, it is cheaper, less time consuming and labour intensive. QF-PCR has been being constantly improved in the last decade as a diagnostic tool for the detection of aneuploidies in prenatal samples.[3–8] The aim of our work was to introduce QF-PCR to our lab and to

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TABLE I. Sets of primers used for QF-PCR[a]

Marker name	Location	Primer sequences 5' to 3'	*n* [b] pmol	Size range bp	Hetero-zygosity
D13S628	13q31–q32	**NED**-TAACATTCATTGTCCCTTACAGAT (F) GCAAGGCTATCTAACGATAATTCA (R)	20	425–465	0.667
D13S634	13q14.3–q22	**6-FAM**–GGCAGATTCAATAGGATAAATAGA (F) GTAACCCCTCAGGTTCTCAAGTCT (R)	15	390–425	0.927
D13S742	13q11–q21.1	**HEX**-ATAACTGGGCTAGGAATGGAAATA (F) GACTTCCCAATTCAGGAGGACT (R)	15	250–290	0.897
D18S380	18q22.3–q23	**NED**-GCATTCTGGGCAACAAAGTGAAAC (F) GAGATAACCCAGGCAAGAACAGGA (R)	10	175–195	0.611
D18S386	18q22.1–q22.2	**HEX**-TGAGTCAGGAGAATCACTTGGAAC (F) CTCTTCCATGAAGTAGCTAAGCAG (R)	20	335–390	0.873
D18S391	18pter–18p11.22	**HEX**-GGACTTACCACAGGCAATGTGACT (F) TAGACTTCACTATTCCCATCTGAG (R)	10	150–170	0.709
D21S11	21q21	**6-FAM**–TTTCTCAGTCTCCATAAATATGTG (F) GATGTTGTATTAGTCAATGTTCTC (R)	10	235–270	0.855
D21S1270	21q21–q22.1	**6-FAM**-CTATCCCACTGTATTATTCAGGGC (F) TGAGTCTCCAGGTTGCAGGTGACA (R)	20	285–330	0.964
D21S1411	21q22.3	GTAGATACATACATATGATGAATGC (F) **NED**-TATTAATGTGTGTCCTTCCAGGC (R)	25	290–335	0.836
AMXY	Xp22.1–22.31 Yp11.2	**NED**-CCCTGGGCTCTGTAAAGAATAGTG (F) ATCAGAGCTTAAACTGGGAAGCTG (R)	2.5	X:105 Y:111	

[a] Fragments were sized using GeneMapper software. F = forward, R = reverse; 6-FAM, HEX and NED are commercial names for fluorescent labels.

[b] Per reaction.

evaluate the usefulness of the chosen marker set in the Croatian population and the reliability and accuracy of the obtained results.

EXPERIMENTAL

Material

Informed consent was obtained from all the patients. Amniocentesis was performed transabdominally under the ultrasound guidance and 15–20 ml of amniotic fluid was acquired; 3 ml was designated for QF-PCR and the rest was used for routine amniotic fluid cell cultivation. All the samples used for QF-PCR were clear, without traces of blood indicating possible maternal contamination and 400 µl of each sample was used for DNA extraction. Apart from amniotic fluid, DNA was also extracted from different samples, including blood, cultivated amniotic fluid cells and paraffin-embedded tissue, all to examine the possibility of using different sample types for the analysis.

DNA Analysis

DNA was extracted from amniotic fluid, blood and cultivated amniotic fluid cells using a JETquick blood DNA spin kit (Genomed GmbH, Germany) and from paraffin-embedded tissue using a DNAeasy tissue kit (Qiagen GmbH, Germany) according to manufacturers' instructions. Microsatellite loci on autosomes 13, 18 and 21 and amelogenin locus were amplified together in a single-assay QF-PCR, using primer sets listed in Table I. PCR reaction was performed essentially as reported by Mann *et al.* (2001), but using Platinum taq polymerase with the accompanying PCR buffer (Invitrogen, UK), and the number of PCR cycles was raised to 28. The reaction products were subsequently separated by capillary electrophoresis on an ABI 3130 genetic analyzer and analyzed with the GeneMapper software. For every sample all loci were called as follows: monoallelic loci were called uninformative while triallelic with 1:1:1 peak area ratios were called trisomic. For diallelic loci, the ratios of shorter to longer allele peak areas were calculated and the loci calls were made: normal for the ratios 0.8–1.4; trisomic for the ratios < 0.65 and > 1.8 and uninformative for the ratios 0.65–0.8 and 1.4–1.8. The samples were defined as trisomic with at least two trisomic loci and normal with at least two normal loci. The exception was the observed gonosomal XYY anomaly defined as trisomic with only one trisomic locus.

RESULTS AND DISCUSSION

The STR loci of all but two DNA extracts from the slow-growing foetal cells were successfully amplified in a single-assay QF-PCR. For the majority of the samples the fluorescent signal level was good, but for a few amniotic fluid samples it was low and hard to evaluate. Therefore,

TABLE II. Types of samples used for QF-PCR and the obtained genotypes[a]

Sample type	Number of samples	Trisomy 18	Trisomy 21	XYY syndrome	Normal
Blood	6	1	2	1	2
Cultivated amniotic fluid cells	9 [b]	1			6
Native amniotic fluid cells	41		2		39
Paraffin-embedded tissue	1				1
TOTAL	57	2	4	1	48

[a] Routine cytogenetic analysis of amniotic fluid and cultivated amniotic cells samples completely confirmed these results (data not shown).

[b] Two of the cultivated amniotic fluid cells samples failed to amplify, probably due to low DNA concentration.

QF-PCR can be successfully applied to different sample types, but a larger starting volume of amniotic fluid is recommended for DNA extraction since raising the number of PCR cycles can lead to unspecific target amplification and high stutter peaks. The results were obtained within 24 hours for the blood, amniotic fluid and cultivated foetal cell samples; 48 hours were needed to obtain results for the paraffin-embedded tissue sample. Out of the total of 57 different samples, 7 aneuploidies were detected, including trisomy 21, trisomy 18 and XYY syndrome (Table II). Different allelic patterns and peak area ratios were observed; trisomic samples showed loci with either triallelic pattern with 1:1:1 peak area ratios or diallelic with approx 2:1 ratio. Normal samples loci were either homozygous or heterozygous with 1:1 peak area ratios (Figure 1). Four normal samples had one diallelic locus with peak area ratios outside the normal range, which was probably caused by the preferential amplification of the one of the alleles in the early cycles of PCR[9] or by the duplication of the particular STR locus.[10] It was also noticed that a DNA quantity can strongly influence diallelic peak area ratios, so repeating the analysis for low quality samples using 3 different DNA concentrations is advisable. Since this assay was not intended as a tool for the detection of sex chromosome aneuploidies, only the amelogenin locus was amplified in order to determine the foetal sex. Therefore, Turner syndrome (X0) cannot be detected, and the detection of sex chromosome complements such as XXY or XYY relies on a single locus only; obviously, more sex loci should be amplified to accurately detect these anomalies. Considering the sample calls for native and cultivated amniotic fluid cell samples, no discordance was found between the results of QF-PCR and routine cytogenetic analysis. This

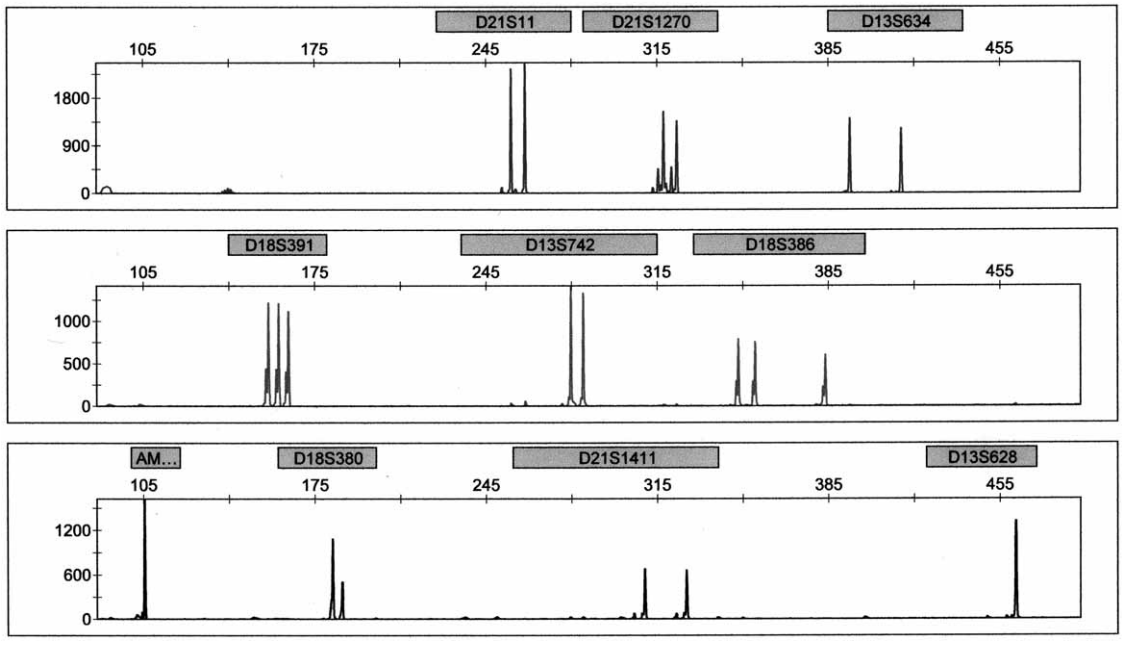

Figure 1. Electopherogram of a female sample with trisomy 18. All the chromosome 21 loci (D21S11, D21S1270 and D21S1411) and 2 of the chromosome 13 loci (D13S634 and D13S742) show a normal diallelic 1:1 pattern. Chromosome 18 loci show characteristic trisomic patterns: triallelic (D18S391 and D18S386) or diallelic with 2:1 ratio (D18S380). Locus D13S628 is homozygous and therefore uninformative. AM = amelogenin locus.

preliminary study with a limited number of samples was aimed at establishing the methodology and its results will serve as a basis for a further analysis of the Croatian population. Much larger studies have already been conducted worldwide with a very low QF-PCR error rate (no amplification or misidentification) and only a minimal discordance between the results of QF-PCR and cytogenetic analysis, similar to our results. In these studies the combined usage of cytogenetic analysis and QF-PCR has completely eliminated failed analyses.[10–11] The chosen marker set was found to have a good potential for usage in the Croatian population since it is a robust system which includes loci with high heterozigosity (Table I) and, therefore, high information content. Similar results have been published by another Croatian group, although not for a completely identical marker set.[12] However, in order to create the ultimate marker set for the Croatian population, a much bigger effort would be needed to determine the heterozygosity of many STR markers examining much more samples.

CONCLUSIONS

Considering the speed and the simplicity of this approach compared with the classical cytogenetic procedure, QF-PCR represents a substantial innovation and a very good solution for the analysis of a large number of samples in a short period of time. Different sample types can be successfully analyzed with the chosen marker set which showed a good potential for the prenatal diagnostics of autosomal aneuploidies in the Croatian population, but more STR loci should be screened to find the most heterozygous ones. Although no discordance was found between the results of cytogenetic analysis and QF-PCR, more samples should be analysed to further evaluate the reliability of the method, especially as a tool for analyzing more complicated samples, including maternally contaminated and mosaic samples. The combined usage of cytogenetic and molecular approach is recommended for the elimination of failed analyses.

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SAŽETAK

Kvantitativni fluorescentni PCR – brza metoda za prenatalnu dijagnostiku najčešćih autosomalnih aneuploidija

Dinko Pavlinić, Snježana Džijan, Feodora Stipoljev, Jasenka Wagner, Goran Ćurić i Gordan Lauc

Autosomne trisomije predstavljaju više od 80 % značajnih kromosomskih poremećaja, a rutinski se utvrđuju kariotipizacijom kultiviranih stanica plodove vode. Nedostatak ovog pristupa je relativno dugo vrijeme potrebno za citogenetičku analizu, kao i nužna visoka stručnost analitičara. Svrha istraživanja bila je uvođenje brže, jednostavnije i jeftinije multipleks QF-PCR metode u naš laboratorij, te procjena informativnosti odabranog seta markera u hrvatskoj populaciji i pouzdanost rezultata dobivenih na ovaj način. STR-lokusi s kromosoma 13, 18 i 21 umnoženi su zajedno, razdvojeni kapilarnom elektroforezom i analizirani. Utvrđeni su karakteristični tripleti i/ili 2:1 alelni omjeri za trisomične uzorke dok su normalni uzorci bili homozigotni ili heterozigotni. Ispitani set lokusa pokazao je visoku heterozigotnost i samim tim dobar potencijal za analizu hrvatske populacije. Rezultati QF-PCR-analize u potpunosti su se podudarali s rutinskom citogenetičkom analizom koja je usporedno izvršena za kultivirane stanice plodove vode.