Rapid detection of chromosome aneuploidies by quantitative fluorescence PCR: first application on 247 chorionic villus samples

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Abstract

We report the results of the first major study of applying quantitative fluorescence polymerase chain reaction (QF-PCR) assays for the detection of major chromosome numerical disorders. The QF-PCR tests were performed on a total of 247 chorionic villus samples, which were analysed blind, without any knowledge of the results obtained using conventional cytogenetic analysis.

The aims of this investigation were to evaluate the detection power and accuracy of this approach by testing a large number of fetal samples and to assess the diagnostic value of each of the chromosome specific small tandem repeat (STR) markers used. In addition, we introduced three more markers specific for chromosomes 13, 18, and X to allow an accurate analysis of samples homozygous for a particular STR. Fluorescent labelled primers were used to amplify 12 STRs specific for chromosomes 21, 18, 13, X, and the amylogenin-like DNA sequence AMXY, expressed on the X and Y chromosomes. In this blind study of 247 fetal samples, 222 were correctly diagnosed by QF-PCR as normal for each of the five chromosomes investigated; 20 were diagnosed by QF-PCR as trisomic for chromosomes 21, 18, or 13, in agreement with the cytogenetic tests. Only one false negative result was observed, probably owing to the mishandling of the sample, which had been transferred through three laboratories before being analysed by QF-PCR. The 247 samples also included four cases of mosaicism or translocation; one case of mosaic trisomy 21 was detected by QF-PCR and the other cases were not identified by QF-PCR.

The results of this investigation provide clear evidence that the QF-PCR assays are powerful adjuncts to conventional cytogenetic techniques and can be applied for the rapid and accurate prenatal diagnosis of the most frequent aneuploidies.

(*J Med Genet* 1999;**36**:300–303)

Keywords: prenatal diagnosis; aneuploidies; quantitative fluorescence PCR

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Received 27 May 1998
Revised version accepted for publication 18 August 1998

Prenatal diagnosis of chromosomal aneuploidies is routinely accomplished by standard cytogenetic techniques. The major disadvantage of these procedures is that fetal cells must be cultured for up to two or more weeks before analysis and that cultivation must usually be prolonged in advanced stages of pregnancy, especially during the third trimester. This time interval between sampling and diagnosis places a considerable emotional burden on the prospective parents.[1] A rapid diagnosis is of paramount importance when an ultrasound examination suggests that the fetus may be affected by an unbalanced chromosome aberration, particularly an autosomal aneuploidy, and a prompt confirmation of the diagnosis is required before taking further action.

For these reasons there is a need for the development of quick methods which would allow rapid detection of major chromosomal abnormalities. The QF-PCR assays, developed for the diagnosis of the most common chromosome disorders, may fulfil this requirement since they can be performed in a very short period of time and can be used for the prenatal diagnosis of aneuploidies involving chromosomes 21, 18, 13, and X.[2–6]

Our aim was: (1) to evaluate the detection power and accuracy of this approach by testing 247 fetal samples in a blind fashion, without previous knowledge of the cytogenetic results; (2) to assess the diagnostic value of additional STR markers which could be particularly useful in the analysis of homozygous samples; and (3) to validate the diagnostic informativeness of each single marker.

Material and methods

Chorionic villus samples (CVS) from 247 women were collected from pregnant women from Italy at 9 to 12 weeks of gestation and tested by conventional cytogenetic analysis in Parma. Small aliquots of the samples were then coded by LT and sent to Graz for QF-PCR analysis. Upon completion of the investigation, the samples were decoded and the results were compared. Conventional cytogenetic tests had shown that 222 had a normal chromosome complement, 21 were trisomic for chromosomes 21, 18, or 13, and four belonged to subjects with mosaicism or translocation (table 1).

DNA was isolated from about 3 mg of a chorionic villus sample following a standard phenol-chloroform extraction procedure.[7] The quantity of genomic DNA used for each PCR assay was between 50 and 200 ng. PCR was carried out in two separate assays, the first set MBPA and B, D21S1414, AMXY, D13S631, and D13S634, and the second set D18S535,

Table 1 Autosomal chromosome aneuploidies present in 247 fetal samples, excluding the translocation case

PCR results	*Trisomy 13*	*Trisomy 18*	*Trisomy 21*	*Mosaic 21*	*Mosaic 18*
True positives	1	4	15	1	0
False negatives	0	1	0	1	1
False positives	0	0	0	0	0

D21S11, D21S1411, D21S1412, and XHPRT. If a homozygous uninformative pattern was found with the markers used to detect abnormalities of a single chromosome, the test was repeated using additional markers (D18S386, D13S258, DXS337; all Genome Data Base).

The markers specific for chromosome 21 were D21S11, D21S1411, D21S1412, and D21S1414, while the markers for chromosome 18 were D18S535, STRs from the myelin basic protein gene (MBP), and D18S386. The primers for the repeats of the MBP gene simultaneously amplify two STR loci (loci A and B). The markers specific for chromosome 13 were D13S631, D13S634, and D13S258 and those specific for the X chromosome were STRs within the hypoxanthine-guanine phosphoribosyl transferase gene (XHPRT) and DXS337. For sexing, primers from an X-Y homologous region of the amelogenin gene (AMXY) on the X chromosome and the amelogenin-like sequence on the Y chromosome were used.

All STR markers were amplified in two separate multiplex PCR assays using the same PCR conditions. The optimised amount of each primer in pmol is given in parentheses after the primer name. Set 1: MBP (20), D21S1414 (2.5), AMXY (2.5), D13S631 (2.5), D13S634 (20). Set 2: D18S535 (5), D21S11 (17.5), D21S1411 (17.5), D21S1412 (5), XHPRT (5). Additional primers: DXS337 (10), D13S258 (10), D18S386 (10).

For each assay PCR amplification was performed in a total volume of 25 µl containing genomic DNA, 200 µmol/l dNTPs, 2.5-20 pmol of each primer, 1 × *Taq* polymerase buffer (1.5 mmol/l $MgCl_2$), and 1.5 units of *Taq* polymerase (Promega, USA). After denaturation at 94°C for five minutes, hot start PCR was carried out for 22 cycles at 94°C for 48 seconds, 60°C for 48 seconds, and 72°C for one minute. Final extension was for five minutes at 72°C.

The PCR products (3 µl) were mixed with 3 µl of loading buffer and 1 µl of Genescan-500 Rox containing the reference molecular size standard. Electrophoretic analysis was performed using a 6% denaturing polyacrylamide gel and using the model 373A DNA sequencer (Applied Biosystems Inc, USA). The amplification products were analysed and their relative fluorescent intensities calculated using Genescan 672 software (Applied Biosystems Inc) as previously described.[5 6 8]

After quantitative analysis of the peak areas the following patterns are expected: in samples from normal subjects, two peaks with a 1:1 ratio or a single peak; in the case of trisomy, either three peaks with a 1:1:1 ratio, a two peak profile with a 2:1 ratio, or rarely a homozygous pattern with a single peak.

Differential fluorescent labelling of primers specific for each chromosome and the different sizes of the PCR products allowed the analysis of several PCR products in two multiplex PCR reactions per chorionic villus sample.

*Table 2 Performance characteristics for QF-PCR results**

	%	*No*
Positive predictive value	100	20/20
Negative predictive value	99.6	222/223
Sensitivity	95.2	20/21
Specifity	100	222/222
False negative rate	4.76	1/21
False positive rate	0	0/223

*Results are based on ploidy analysis by QF-PCR for chromosomes 13, 18, and 21, as compared with cytogenetic analysis. Cases with mosaicism or translocation were excluded.

Results

ACCURACY

Trisomy 21 was diagnosed by QF-PCR assays in 15 cases, trisomy 18 in four cases, and trisomy 13 in one case (table 1). The correct diagnosis of these aneuploidies was confirmed by cytogenetic analysis and no false positive results were observed. However, one sample, diagnosed as trisomic for chromosome 18 by cytogenetic tests, appeared to be normal disomic when investigated by QF-PCR. Since further PCR amplifications, using several primers for chromosome 18, failed to detect the aneuploidy, this abnormal result was attributed to a possible mishandling of the sample which had been transferred through several laboratories before reaching Graz.

Only one sample out of three cases with mosaicism for chromosome 21 or 18 was detected by QF-PCR. QF-PCR assays are based on quantitative ratios of fluorescent PCR products, so this approach is unsuitable for the detection of small subpopulations of aneuploid cells in a single sample. As expected, a case of balanced translocation was not recognised by QF-PCR, since the current protocol is not designed for these chromosome abnormalities.

Sexing of all samples was performed by PCR amplification of X and Y chromosome derived sequences. The sex was correctly identified in all but two samples from chromosomally normal fetuses. One male sample was misdiagnosed as female and one female sample was incorrectly sexed as male. Unfortunately we were not able the retest these samples because we received only a small aliquot of each and could not obtain any additional chorionic villi from the same patients to repeat the experiment. Since these two samples were coded sequentially, the most probable explanation is that they had been mishandled.

Table 2 describes the performance characteristics for PCR results.

UNINFORMATIVE RESULTS

All samples (n=247) were heterozygous and thus informative for at least one marker specific for chromosomes 21 and 18. One sample showed a homozygous uninformative pattern with all three markers specific for chromosome 13 (D13S631, D13S634, D13S258); 10 sam-

Table 3 Frequency of homozygous, and thus uninformative, pattern of the STR markers

Marker	*Normal samples (n=227*) homozygous*	*Trisomic samples (n=20) homozygous*
Chromosome 21 marker		
D21S11	39 (17.2%)	1 (5%)
D21S1414	39 (17.2%)	1 (5%)
D21S1411	26 (11.5%)	2 (10%)
D21S1412	38 (16.7%)	1 (5%)
Chromosome 18 marker		
D18S535	54 (23.8%)	6 (30%)
MBPA	53 (23.3%)	8 (40%)
MBPB	51 (22.5%)	5 (25%)
Chromosome 13 marker		
D13S631	47 (20.7%)	3 (15%)
D13S634	33 (14.5%)	2 (10%)
X chromosome marker		
XHPRT	29/116 (25%)	1/8 (12.5%)

*Cases with mosaic and translocation (n=4) and one false negative sample were included.

ples were homozygous for all chromosome X markers (XHPRT, DXS337).

The frequency of homozygous and thus uninformative patterns are described in table 3.

Discussion

This is the largest blind study performed so far in which chorionic villus samples have been tested using STRs and QF-PCR for the prenatal detection of chromosome 21, 18, and 13 trisomies, while sexing the fetuses with markers specific for the X and Y chromosomes. In addition to our initial approaches, the diagnostic efficiency of this test was extended using three more markers specific for chromosomes 18, 13, and X. Using these 12 markers, all samples were found to be informative for at least one STR marker specific for chromosomes 21 and 18.

Occasionally, one of the markers used in a multiplex assay may produce equivocal results of the diallelic peaks; in these cases the samples should be reanalysed by testing the marker in a separate reaction. In agreement with previous observations,[8 9] the marker most prone to produce equivocal STR ratios was the MBP for locus A on chromosome 18. However, in the present study, the other chromosome 18 markers allowed us to reach the correct diagnosis in all samples investigated.

In this study, as in previous investigations when a relatively large amount of DNA was analysed by QF-PCR,[2 3 5] the ratio of the two peaks of fluorescence in normal heterozygous samples was close to 1:1 and no preferential amplification of one allele was observed. Allelic drop out (ADO) is a rare event, which may occur when single cells are tested; it can be avoided by an appropriate method of DNA extraction[10] and did not seem to have affected the present results.

The chromosome 21 markers D21S1414 and D21S11 amplify the same repeat sequence. Despite the homology of these two markers they have both been used, since in this series, as well as in other studies (J Sherlock, M Adinolfi, unpublished results), some samples from normal subjects produce diallelic peaks differing by only 2 bp instead of the expected 4 bp. Because in these cases it could be difficult to distinguish between heterozygosity or homozygosity of the pattern, the use of both markers with different size products provides additional information.

One false negative sample was observed; this chorionic tissue appeared to be derived from a fetus with trisomy 18 when tested by conventional cytogenetic analysis, but produced a clear normal heterozygous (diallelic) pattern when repeatedly analysed by QF-PCR and the chromosome 18 specific markers D18S535 and MBPA and B. Since this specimen was first tested by conventional methods in Parma (Italy), then part of it sent to Milan, and finally a small aliquot coded and posted to Graz, we assume that the misdiagnosis was the result of a mistake during the handling of the sample. Gross maternal cell contamination of the sample could have been ruled out as a potential cause of this finding by checking for the presence of a paternal allele.

This series included three cases of mosaicism involving chromosomes 21 and 18; when tested by QF-PCR, one sample showed a triallelic pattern while the low mosaicism of the other two samples could not be detected. Since QF-PCR assays performed with the present STR markers is not designed to detect low levels of mosaicism, these results do not invalidate the clinical diagnostic value.

The diagnosis by QF-PCR of X chromosome abnormalities, such as Turner syndrome (45,X), is still hampered by the low polymorphism of several X chromosome markers. However, work in progress suggests that this problem could be solved by the use of a recently discovered marker in the Xq/Yq pseudoautosomal region (V Cirigliano, M Adinolfi, personal communication).

QF-PCR is suitable for detecting all non-mosaic aneuploidies. However, the current design of the QF-PCR assays can only detect major chromosome abnormalities. Since they comprise the majority of all viable fetal aneuploidies, this limitation has little negative diagnostic impact. A further advantage of QF-PCR tests is that they can be readily performed on a small number (5-10) of cells in multiplex assays without observing allelic preferential amplification or ADO.[10]

Alternative approaches for rapid cytogenetic analysis, like rapid karyotyping and FISH on uncultured amniocytes, have been developed recently. However, rapid karyotyping is restricted to highly specialised laboratories. FISH analysis, on the other hand, is a labour intensive and time consuming technique which potentially limits its application for a high throughput of samples.

Using the present STR markers, the QF-PCR assay is not a substitute for conventional cytogenetic analysis. It provides a rapid and economical method for the prenatal diagnosis of major chromosome defects using amniotic fluid cells[2 5 6] or, as shown in this paper, chorionic tissue. The advantages of this approach are: (1) the reduction of parental anxiety through the rapid exclusion of numerical chromosome aberrations in fetuses, thus positively affecting the maternal bond; (2) the rapid detection of major aneuploidies particularly in fetuses suspected of carrying chromosome dis-

orders on the basis of ultrasound or biochemical test; and (3) the possibility of performing a termination of pregnancy at an early stage of gestation, when the result clearly indicates the presence of fetal chromosome disorder. The results of the present study support the use of QF-PCR as an accurate adjunct to conventional cytogenetic techniques, which allows detection of common aneuploidies in a short period of time.

This work was supported by grant No P10470-Med from the Austrian Science Foundation, Vienna (FWF). We would like to thank Dr Lamberto Camurri and colleagues (HI-TECH, Parma, Italy) for cytogenetic analyses of the samples. We thank Professor G Kostner and his team (Department of Biochemistry, University of Graz) for the use of the DNA sequencer 373A (Applied Biosystems Inc, USA) for electrophoretic analysis.

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