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Rapid prenatal diagnosis of common chromosome aneuploidies by QF-PCR. Assessment on 18 000 consecutive clinical samples

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The quantitative fluorescent PCR (QF-PCR) assay, introduced during the last few years, allows prenatal diagnoses of common chromosome aneuploidies in a few hours after sampling. We report the first assessment of QF-PCR performed on a large cohort of 18 000 consecutive clinical specimens analysed in two different Centres. All samples were analysed by QF-PCR using several selected STR markers together with amelogenin and, occasionally, SRY for fetal sexing. Results were compared with those obtained by conventional cytogenetic analysis. In 17 129 tests, normal fetuses were detected by QF-PCR. No false positives were observed. All 732 cases of trisomy 21, 18, 13, triploidies, double trisomies as well as all but one fetuses with X and Y aneuploidies were correctly diagnosed. Chromosome mosaicism could also be suspected in several samples. In some cases of *in vitro* culture failures, QF-PCR was the only evidence of fetal X, Y, 21, 18 and 13 chromosome complement. QF-PCR proved to be efficient and reliable in detecting major numerical chromosome disorders. The main advantages of the molecular assay are its very low cost, speed and automation enabling a single operator to perform up to 40 assays per day. QF-PCR relieves anxiety of most parents within 24 h from sampling and accelerates therapeutic interventions in the case of an abnormal result. In countries where large scale conventional cytogenetics is hampered by its high cost and lack of technical expertise, QF-PCR may be used as the only prenatal diagnostic test.

Key words: aneuploidy/QF-PCR/rapid prenatal diagnosis/STR

Introduction

Prenatal diagnoses of chromosome abnormalities are performed by conventional cytogenetic analysis using *in vitro* culture of fetal nucleated cells retrieved by amniocentesis, chorionic biopsy or fetal blood sampling. Improvements in culture media have only partially reduced the interval between the collection of the samples and the reporting of the results. This is a very anxious time for the parents, particularly if non-invasive screening tests (biochemical and/or ultrasound), performed during the first trimester of pregnancy, have suggested an increased risk of chromosome disorders (Sjogren and Uddenberg, 1990; Marteau *et al.*, 1992). The need for rapid prenatal diagnoses prompted the use of the fluorescence *in situ* hybridization assay (FISH) for the detection of major chromosome aneuploidies using uncultured cells (Ward *et al.*, 1993; Adinolfi and Crolla, 1994; Eiben *et al.*, 1998). However, the cost of this labour-intensive procedure has limited its application mainly to high-risk pregnancies (Evans *et al.*, 1999; Pergament *et al.*, 2000; Tepperberg *et al.*, 2001). During the past 10 years, quantitative fluorescent PCR (QF-PCR) has been introduced to perform rapid prenatal diagnoses of common chromosome aneuploidies (Mansfield 1993; Pertl *et al.*, 1994, 1996; Adinolfi *et al.*, 1997, 2000). The clinical utility of this assay has repeatedly been confirmed together with its high sensitivity and specificity in detecting major chromosome abnormalities (Pertl *et al.*, 1999a,b; Schmidt *et al.*, 2000; Adinolfi and Sherlock, 2001; Cirigliano *et al.*, 2001a,b; Levett *et al.*, 2001; Mann *et al.*, 2001). One of the advantages of QF-PCR is the automation of part of the procedure that allows high throughput of samples at a very low cost (Adinolfi *et al.*, 2000; Adinolfi and Sherlock, 2001).

QF-PCR tests are now performed in several prenatal Centres in Europe for the detection of major numerical abnormalities affecting chromosomes X, Y, 21, 18 and 13, with results provided in 24 h (Shmidt *et al.*, 2000; Cirigliano *et al.*, 2001b, 2002, 2003; Mann *et al.*, 2001; Voglino *et al.*, 2002; Bili *et al.*, 2002; Andonova *et al.*, 2004).

Here we report the results of screening 18 000 consecutive fetal samples, using both QF-PCR and conventional cytogenetic analysis.

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Materials and methods

Samples were collected between December 1999 and October 2003 in two different genetic Centres (General Lab, Barcelona, Spain and Sant' Anna Hospital, Turin, Italy). Both institutions were also offering rapid QF-PCR service to other cytogenetic laboratories, private clinics and public hospitals, so that several high-risk pregnancy samples were received nationwide on a daily basis.

The majority of prenatal samples (93%) were amniotic fluids (16 746) collected between 12 and 31 weeks of gestation; 264 samples were heavily bloodstained and suspected of being contaminated with maternal cells; these samples were analysed together with a maternal buccal wash or blood sample in order to distinguish between maternal and fetal STR profiles.

A total of 625 chorionic villous samples (CVS, 3.5%), collected between 11 and 13 weeks of gestation, 123 fetal blood samples (0.7%) and 506 fetal tissues from aborted fetuses (2.8%) were also investigated.

In 146 prenatal cases, QF-PCR was not requested at the time of collecting the samples, but it was only performed afterwards on cytogenetic cultures. These included slow *in vitro* growing samples and failed cultures contaminated by bacterial or fungal cells; QF-PCR was also performed in some cases of long-term CVS cultures and cultured amniotic fluids to assess the fetal origin of the predominant cell population.

The most frequent indication for the request of rapid QF-PCR diagnosis of common chromosome aneuploidies was advanced maternal age, followed by abnormal serum screenings, parental anxiety and abnormal ultrasound findings.

All women received genetic counselling, including detailed information on the advantages and limitations of the rapid QF-PCR assay; routine informed consent was obtained in all cases.

Genomic DNA was extracted from 0.5–1 ml amniotic fluids or cell cultures, 5 µl of fetal bloods, 200 µl buccal washes, a small villous fragment or 1–2 mm tissue sections using a resin-based procedure (Instagene Matrix; Bio-Rad Laboratories, USA), as previously described (Cirigliano *et al.*, 2001b). QF-PCR was performed in both Centres using the large set of STR markers for chromosomes X, Y, 21, 18 and 13 listed in Table I. Primer sequences, retrieved from The Genome Data Base (http://www.gdb.org) and Cooperative Human Linkage Center (http://www.chlc.org), were used for multiplex QF-PCR without modifications. A total of six STR for the X and Y chromosomes, six for chromosome 21, five for chromosome 18 and four for chromosome 13 were selected because of their high level of heterozygosity, thus reducing the number of uninformative samples (Cirigliano *et al.*, 1999, 2001a,b). Furthermore, non-polymorphic sequence of the amelogenin (AMXY) gene and, occasionally, SRY were also included to detect fetal sex.

Table I. Markers used for QF-PCR detection of chromosomes X, Y, 21, 18 and 13 copy number

Marker	Labelling	Allele range (bp)	Chrromosome location
SRY	6-FAM	Y 463	Yp11.3
AMXY	6-FAM	X 106 Y 112	Xp/Yp
X22	6-FAM	189–242	Xq28Yq (PAR2)
HPRT	6-FAM	268–296	Xq26.1
DXS6803	HEX	113–128	Xq
DXS6809	HEX	240–278	Xq
SBMA	NED	144–180	Xq11.2-Xq12
DXS8377	HEX	203–245	Xq28
D21S11	6-FAM	214–242[a]	21q21
D21S1435	HEX	163 – 187	21q21
D21S1446	PET	220–260	21q22.3-ter
D21S1414	6-FAM	334–362[a]	21q21
D21S1411	HEX	266–319	21q22.3
D21S1412	6-FAM	384–414	21q22.2
D18S858	6-FAM	150–240	18q21.1
D18S391	HEX	130–190	18pter-18p11.22
D18S51	PET	271–331	18q21.33
D18S535	NED	126–156	18q12.2
D18S386	NED	330–387	18q22.1
D13S631	VIC	192–218	13q31-32
D13S634	HEX	464–490	13q14.3
D13S258	NED	230–267	13q21
D13S742	HEX	235–315	13q11-q21

Different labelling of primers amplifying products of similar size allows multiplex PCR reactions.
[a]D21S11 and D21S1414 amplify the same STR locus with a different length of the amplicon of 122 bp.

All STR were stable tri-, tetra- and penta-nucleotides, suitable for multiplex PCR because of their minimal production of artefacts during amplification. The STR locations along the examined chromosomes were also taken into account in order to increase the possibility of detecting partial trisomies (Figure 1). All forward primers (Roche-TIB Molbiol, Germany) were labelled with fluorescent molecules allowing accurate sizing and quantification of QF-PCR products. Primers producing amplicons of similar sizes were labelled with different fluorochromes in order to be amplified and analysed in the same multiplex QF-PCR reactions (Sherlock *et al.*, 1998).

In the course of this study the two Centres developed different combinations of primers in multiplex QF-PCR reactions in order to simplify sample handling and data analysis (Cirigliano *et al.*, 2001a,b, 2002; Voglino *et al.*, 2002).

Fetal sex and chromosomes X and Y copy number were assessed for all cases by amplification of the homologous gene AMXY together with the pseudoautosomal X22 and chromosome X-specific markers (Table I) (Cirigliano *et al.*, 1999, 2001a,b). In the course of this study the D21S1411 STR was used as internal control to quantify the X-linked HPRT marker in order to assess X chromosome copy number in all samples (Cirigliano *et al.*, 2002).

Aneuploidy screenings were initially performed (almost the first 6000 samples) using two multiplex QF-PCR assays that included two STR on chromosomes X, 21, 18, 13, and the AMXY. Samples showing homozygosity for two chromosome-specific markers, as well as all aneuploid cases, were re-tested with chromosome-specific multiplex PCR using up to four STR on chromosome 21, four STR on the X, and three markers on each chromosome 18 and 13 (Cirigliano *et al.*, 2001b). The following group of ~12 000 samples was tested adding two more STR specific for chromosomes X and 18 and one more marker for chromosome 13 (Table I).

The fluorescent QF-PCR products and size standards were analysed by capillary electrophoresis on ABI 310 automated DNA sequencers and GeneScan 3.7 Software (Applied Biosystems, USA) as previously described (Pertl *et al.*, 1996; Adinolfi *et al.*, 1997; Cirigliano *et al.*, 2001a, 2002). In both Centres, after the first 18 months of activity of the QF-PCR service, this model of automated DNA sequencer was replaced with a four-capillary instrument (ABI 3100-Avant; Applied Biosystems) in order to increase sample throughput. Final data were generated using GeneScan and Genotyper Softwares (Applied Biosystems) as previously described (Adinolfi *et al.*, 1997; Pertl *et al.*, 1994, 1996; Cirigliano *et al.*, 1999, 2001a,b, 2002, 2003).

Normal chromosome complement was assessed by detecting, for the correspondent STR, two different alleles with a ratio between fluorescent peak areas of 1:1 (ranging between 0.9 and 1.3:1). Trisomies were detected, with the correspondent chromosome specific STR, either as three peaks of fluorescent activity with area ratio close to 1:1:1 or as two unbalanced peaks with area ratio of 2:1 (ranging from 1.7 to 2.4:1) (Adinolfi *et al.*, 1997, 2000; Cirigliano *et al.*, 1999, 2001a,b). The presence of trisomic triallelic or diallelic patterns for at least three different STR on the same chromosome was considered as evidence of trisomy. The same trisomic patterns observed for all chromosome-specific STR were indicative of triploidy. All prenatal samples were processed and reported within 24–48 h; in 98% of cases results were made available within the next working day.

Conventional cytogenetic analyses were performed on all prenatal samples, cultured and harvested according to standard procedures; depending on the specimen, the results were issued between 5 and 28 days (mean reporting time of 2 weeks for amniotic fluids).

Results

DNA extraction and QF-PCR amplification were successful in 17 986 cases (99.9%). Only 14 samples failed to amplify; all these cases were failed cytogenetic cultures heavily contaminated by

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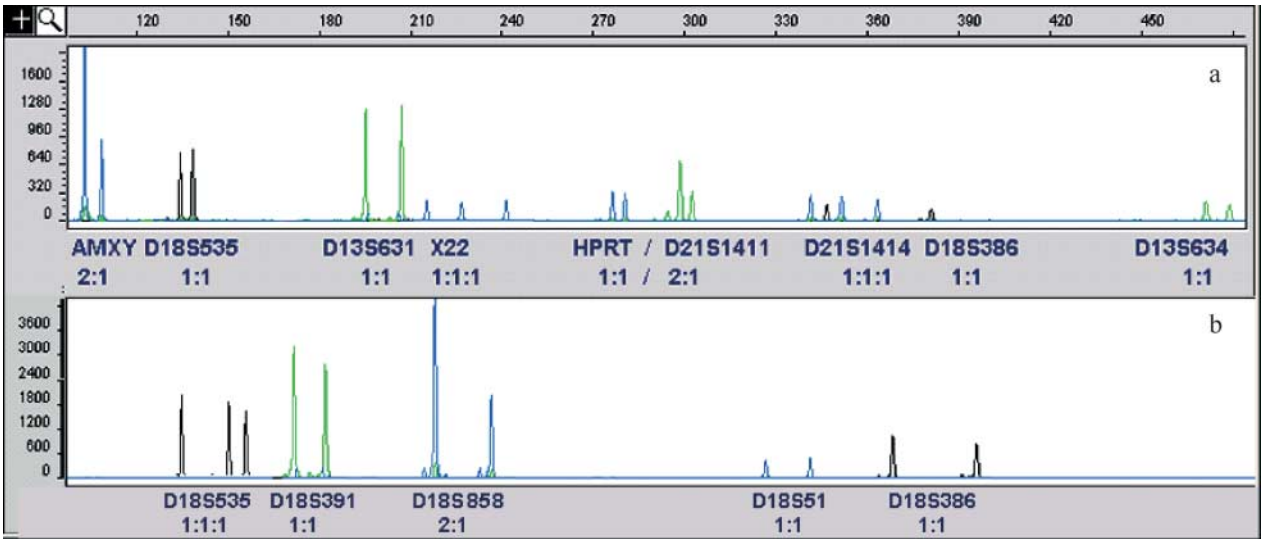

Figure 1. (**a**) Electrophoretogram showing the QF-PCR detection of a 48,XXY + 21 fetus. The X-specific peak of the AMXY is in double dose if compared to the Y; the presence of three sex chromosomes is confirmed by the trisomic triallelic pattern of the X22; two X chromosomes are also detected by the HPRT marker. Trisomy 21 is documented by the trisomic patterns observed for both STR used for this chromosome. (**b**) QF-PCR detection of partial trisomy 18. Two out of five chromosome 18-specific STR have trisomic patterns; D18S858 is compatible with duplication but the three different alleles of D18S535 show that the structural anomaly resulted from an insertion between 18q12.2 and 18q21.1.

bacterial or fungal cells. These samples had not been analysed by QF-PCR soon after collection since the molecular tests were requested by cytogeneticists only after cell culture failures. Conventional cytogenetic analysis was not achieved in a total of 26 prenatal cases because of cell culture failures (24 samples) or maternal cell overgrowth (two cases). In 12 cases QF-PCR tests were successful and gave normal results; thus, a second invasive procedure could be avoided. In 111 bloodstained amniotic fluids, QF-PCR showed the presence of a high level of maternal cell contamination and no result could be achieved other than fetal sex.

Detection of normal samples

The results of rapid aneuploidy screenings and conventional cytogenetic analyses are reported in Table II. In 17 052 samples the QF-PCR tests were informative for the five chromosomes examined and provided evidence that fetuses had normal chromosome complement. The detection of heterozygous patterns with fluorescent peak ratios close to 1:1 for at least two chromosome-specific STR was sufficient to perform the diagnosis. No false positive results were observed.

Seventy-seven cases (0.4%) were uninformative for copy number of either chromosome 18 or 13, due to the homozygosity of the employed polymorphic markers. These samples were later diagnosed as normal for these chromosomes by conventional cytogenetic analysis. However, 64 of these uninformative samples (88%) were tested using only three markers on chromosomes 18 and 13 during the first 2 years (~6000 samples). To reduce the number of uninformative results, two more STRs specific for chromosome 18 and one for chromosome 13 were selected. The inclusion of these markers markedly reduced the frequency of uninformative samples to nine in the next ~12 000 samples.

Detection of fetal sex

Fetal sex and chromosome X and Y copy number were determined in all cases by amplification of the non-polymorphic sequences of the amelogenin gene (AMXY). QF-PCR of this marker generates chromosome X- and Y-specific products differing 6 bp in length that can be identified and quantified (Figure 1). The results were in agreement with cytogenetic analysis in 17 972 cases (99.9%). In two prenatal samples, amplification of the AMXY marker resulted in apparently normal female fetuses that were later diagnosed as males

Table II. Results of testing 18 000 consecutive fetal samples by QF-PCR and conventional cytogenetic analysis

Karyotype	Cytogenetics	QF-PCR
46,XX; 46,XY	17174	17129
47,XX +21; 47,XY + 21	344[a]	344
47,XX +18; 47,XY + 18	162	162
47,XX + 13; 47,XY + 13	61	61
69,XXX; 69,XXY	51	51
45,X	56	55
47,XXY	29	29
47,XYY	20	20
47,XXX	4	6[b]
49,XXXXX	1	1
49,XXXXY	1	1
48,XXY + 21	1	1
48,XXY + 18	1	1
Mosaics	25	12
Other aneuploidies	19	–
Structural balanced	18	–
Structural unbalanced	7	2
Maternal contamination[c]	2	111
Failed	24	14
Total abnormalities	800	746
Sensitivity[d] (%)		93.2
Specificity[e] (%)		100
PPV[f] (%)		100
NPV[g] (%)		99.7

[a]Seventeen cases of unbalanced Robertsonian translocations.
[b]Two cases diagnosed as 47,XXX by QF-PCR were mosaics 46,XX/45,X.
[c]Only maternal cells grown in culture or heavily contaminated with maternal DNA.
[d]Sensitivity is the percentage of abnormal karyotypes detected by QF-PCR.
[e]Specificity is the percentage of unaffected fetuses detected by QF-PCR.
[f]Positive predictive value (PPV) is the probability of a fetus with positive QF-PCR result to be confirmed as aneuploid by cytogenetic analysis.
[g]Negative predictive value (NPV), is the probability of a fetus with normal QF-PCR result to be confirmed as normal by cytogenetic analysis.

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by cytogenetic analysis. Further investigations showed that the discordant QF-PCR results were due to a Y chromosome microdeletion that included the AMXY, but not the SRY, DNA sequences. In one case this rare abnormality could be demonstrated by QF-PCR assay as inherited from the father.

The Y-specific product of the AMXY marker was found duplicated in two normal males that produced fluorescent peaks in a ratio of 2:1 similar to that observed in 47,XYY males. In both cases, the correct diagnosis was achieved by the simultaneous amplification of the X22 marker that confirmed the presence of a single Y chromosome.

The X chromosome-specific product of the amelogenin was also found duplicated in three normal males; in these cases, the QF-PCR of this sequence generated fluorescent peaks with ratios of 2:1, similar to those observed in patients with Klinefelter syndrome (47,XXY). However, in all these cases the correct diagnosis could be performed by the simultaneous amplification of five X chromosome STRs and the pseudoautosomal X22 marker that allowed the correct assessment of normal XY chromosome complements.

Detection of aneuploidies

In the present series, 800 abnormal karyotypes were diagnosed by conventional cytogenetic analysis. A total of 746 aneuploidies was also detected by the rapid QF-PCR assay (93.3% sensitivity) within 24 h from collection of the samples (Table II). The rapid test demonstrated 100% specificity for chromosomes X, Y, 21, 18 and 13 aneuploidies with positive predictive value (PPV) of 100% and negative predictive value (NPV) of 99.7%.

Detection of autosomal trisomies

All 618 samples with trisomies of chromosomes 21, 18 or 13 and triploidies were readily diagnosed by the multiplex QF-PCR assay without false negative results (100% sensitivity and specificity). A total of 344 fetuses with trisomy 21, 162 with trisomy 18, 61 with trisomy 13 and 51 triploidies were identified in this series (Table II).

Two cases of partial trisomies involving chromosome 18 [46,XY.dup(18)(q12.2q21.2) and 46,XYdup(18)(q11.2q22)] were also diagnosed by detecting trisomic patterns for two out of the five markers used for this chromosome (Figure 1).

As shown in Table II, only 19 cases of numerical abnormalities were not detected by QF-PCR; they involved chromosomes for which specific STRs were not included, such as trisomies of chromosomes 22, 16, 15 and small extra chromosome markers.

STR duplications

Submicroscopic polymorphic duplications of microsatellites were observed in 19 cases as clear trisomic triallelic or diallelic patterns for one chromosome-specific STRs (Table III, Figure 2). All these samples were diagnosed as normal by cytogenetic analysis. Duplications were observed in three samples for one STR on chromosome 21, in nine cases for one of the markers selected on chromosome 13; in six samples in the pseudoautosomal X22 and in the remaining case with the D18S386 marker.

The maternal origin of the duplication could be demonstrated in nine cases by QF-PCR analysis of the same marker in both parents; in six samples the polymorphism was found as inherited from the father (Table III, Figure 2). In two out of 19 samples the duplication was found to be *de novo*; in both cases paternity was confirmed by amplification of all other available STR markers. In the remaining two cases, parental DNA was not available for further investigations. Three more normal samples (one CVS, one amniotic fluid and one maternal blood) produced unbalanced triallelic patterns for a single marker consistent with somatic microsatellite instability (Mann *et al.*, 2003). In two cases the D18S386 was involved and in the remaining one the HPRT marker. However, these three samples were analysed using other informative chromosome-specific STR that allowed correct prenatal diagnoses.

Table III. Submicroscopic duplications of STR markers observed in 19 prenatal samples

	Paternal	Maternal	Unknown	*De novo*	Total
D13S631	3	3	1		7
X22	1	4	1		6
D13S634	1	1			2
D21S1411	1			1	2
D21S1414		1			1
D18S386				1	1
	6	9	2	2	19

The frequency of this polymorphism is extremely low (0.1%).

Detection of sex chromosome aneuploidies

The diagnosis of Turner syndrome was based on the detection of a single QF-PCR product for all the X chromosome polymorphic markers, in the absence of the Y-specific amelogenin product (Cirigliano *et al.*, 1999, 2000, 2001a,b, 2002). The QF-PCR detection of Turner's syndrome was successful in 55 out of 56 cases (Table II). The only false negative QF-PCR result in our series was a 45,X fetus that was diagnosed as normal female. In this case, three out of four employed STRs produced single PCR peaks, while the X22 marker suggested that the sample was derived from a normal heterozygous female; this X22 pattern probably resulted from a polymorphic submicroscopic duplication of this STR sequence (Figure 2). In the course of this study, the introduction of D21S1411 used as internal control to quantify the X-linked HPRT marker (Cirigliano *et al.*, 2002) and the inclusion of up to six STR on the X chromosome, further improved the detection of X monosomy. This approach also allowed the prenatal detection of rare normal females homozygous for up to four STR markers. As shown in Table II, two out of six samples diagnosed as 47,XXX by QF-PCR showed discordant results when analysed by conventional cytogenetic tests. The first amniotic fluid resulted in a 45,X karyotype. This discordant result suggested the possibility of a mosaic 45,X/46,XX with the normal cell line not grown during *in vitro* cultures (Figure 3). Amniocentesis was repeated together with fetal blood sampling (FBS) and the suspected X chromosome mosaicism was confirmed by cytogenetics analysis of the amniotic fluid; however, only normal cells were detected in the fetal blood sample. In the second case of amniotic fluid, diagnosed as trisomy X by QF-PCR, cytogenetic analysis documented the presence of a mosaic fetus 45,X[75]/46,XX[25].

All 53 non-mosaic aneuploidies involving both the X and Y chromosomes, including 47,XXY, 47,XYY and rare karyotypes such as X,Y pentasomies or double trisomies were correctly diagnosed by QF-PCR (Table II and Figure 1).

Detection of chromosome mosaicisms

QF-PCR was effective in detecting 12 out of 25 fetuses with chromosome mosaicism (Table II). The presence of two different cell lines in the prenatal samples was detected in all cases of 45,X/46,XY mosaicism (Figure 3). In two of these cases, the presence of a cell line with the Y chromosome could only be

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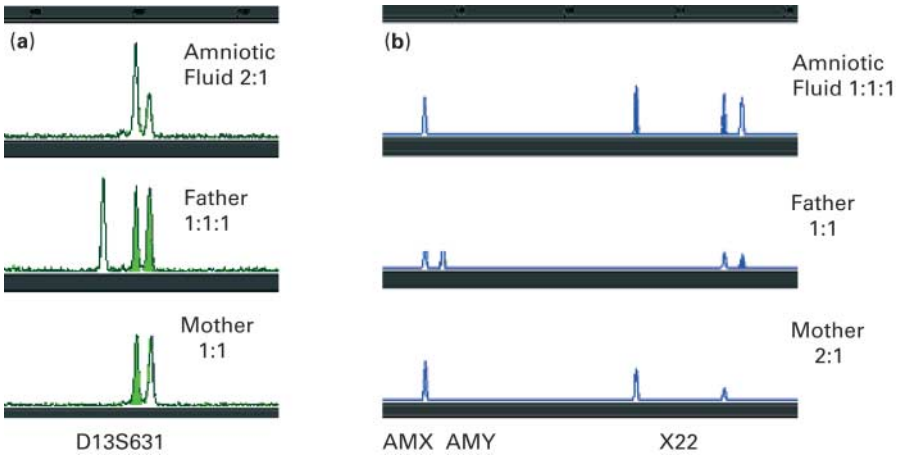

Figure 2. (**a**) Submicroscopic duplication of D13S631 marker. The trisomic diallelic pattern observed in the normal fetus is due to the inheritance of a duplicated paternal sequence with two alleles in 1:1 ratio (highlighted) together with a maternal allele of the same size. (**b**) Submicroscopic duplication of the X22 marker. The STR sequence is duplicated in the mother. Two alleles (highlighted) are transmitted to the fetus on one maternal X chromosome, another allele is inherited from the father (highlighted), generating the trisomic triallelic pattern observed in the normal female fetus.

demonstrated by the QF-PCR detection, in the uncultured samples, of a small Y-specific fluorescent PCR product of the AMXY; in these two cases cytogenetic analysis resulted in pure 45,X. In three fetal samples, a mosaicism 45,X/46,XX could be suspected by QF-PCR as unbalanced allele ratios for the employed X chromosome markers (Figure 3). Chromosomes 21 and 18 mosaic could be diagnosed in two and one cases respectively as small extra alleles for at least two chromosome-specific STRs (Figure 3). In agreement with previous studies (Cirigliano *et al.*, 1999, 2001a,b; Schmidt *et al.*, 2000), low level chromosome mosaicisms could not be detected by QF-PCR in the remaining cases.

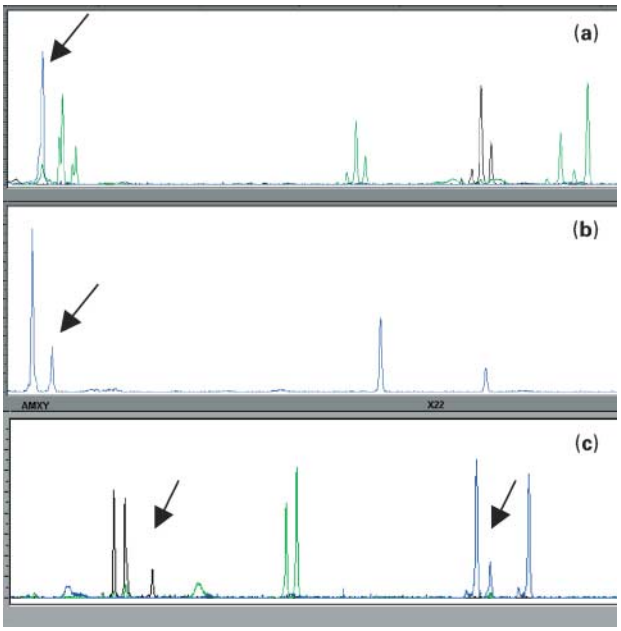

Figure 3. (**a**) QF-PCR detection of a mosaic fetus 45,X/46,XX. Fetal sex is assessed by the presence of the X chromosome-specific product of the AMXY (arrow). Normal and aneuploid cell lines are in the same proportion, generating 2:1 peak ratios in four X-linked STR. (**b**) QF-PCR detection of a mosaic fetus 45,X/46,XY. The low proportion of 46,XY cell line is detected as a small Y chromosome-specific product of the AMXY (arrow) and a small extra allele of the pseudoautosomal X22 marker. (**c**) QF-PCR detection of mosaic trisomy 18. The extra chromosome is detected either as a small extra allele for two STRs (arrows) or as a skewed ratio in the third chromosome 18 marker.

Analysis of samples with maternal cell contamination

Rapid diagnosis of aneuploidies by QF-PCR may be hampered by the presence of contaminating maternal cells in the samples, but in the present series 264 heavily bloodstained amniotic fluids were also analysed (Table IV). Normal fetuses were diagnosed in 151 cases. The prenatal diagnosis of normal female fetuses was made possible by testing the corresponding maternal DNA with the same STR markers. In samples retrieved from male fetuses, quantification of maternal cell contamination was possible by comparing fluorescence of the X-specific AMXY sequence with the purely fetal Y product.

Two aneuploidies were also identified in this subset of samples (Table IV). In the remaining 111 bloodstained amniotic fluids only the diagnosis of fetal sex was possible by detecting Y-specific chromosome sequences.

Cytogenetic analysis was successfully performed in 262 out of 264 bloodstained samples; it could also diagnose three aneuploidies that were undetectable by QF-PCR because of a very high level of maternal cell contamination (Table IV). In two samples, only maternal cells were present in the *in vitro* culture. However, in both cases the QF-PCR detection of Y sequences in the uncultured samples revealed the culture artefact and allowed correct sexing of the fetus.

According to previous studies (Cirigliano *et al.*, 2003), the STR markers selected for multiplex QF-PCR assays were informative for the assessment of zygosity in all 184 multiple pregnancies analysed in the course of this study.

Table IV. Results of testing 264 bloodstained amniotic fluids

Karyotype	Cytogenetics	QF-PCR
46,XX[a]-46,XY	257	151
47,XXY	1	
47,XX + 21; 47,XY + 21	3	1
47,XX + 13	1	1
Uninformative	2	111

[a] Fetal origin of the cells was established for all normal female fetuses by analysing maternal DNA with the same STR markers.

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Discussion

The analysis of this large series of prenatal diagnoses (18 000 consecutive clinical specimens), performed by both the QF-PCR assay and conventional cytogenetics, should help to assess the advantages of applying the molecular technique on fetal samples collected by amniocentesis, chorionic biopsy and cordocentesis. The multiplex QF-PCR tests were based on the use of selected STR markers that could detect the most frequent chromosome disorders.

The overall results show that QF-PCR is a rapid, simple and accurate diagnostic test. In 17 129 normal pregnancies, which were correctly diagnosed by QF-PCR, without false positive results, parents could be informed about the outcome of the test within 24–48 h from the collection of the samples. Thus, the rapid assay reached the purpose of greatly reducing the anxiety in 95% of all parents waiting for full karyotype analysis. QF-PCR performed on clear amniotic fluids correctly diagnosed 100% of trisomies for chromosomes 21, 18, 13, tripolidies and non-mosaic aneuploidies involving both chromosomes X and Y without false negative results. A total of 746 aneuploidies, detected by QF-PCR, accounts for 93.2% of fetuses with abnormal karyotypes diagnosed by conventional cytogenetic tests. The sensitivity of the molecular assay increased up to 95.4% if only clinically significant cytogenetic abnormalities were taken into account. No false positives were observed and, in several cases, the great efficiency and reliability of QF-PCR allowed early termination of affected fetuses without further waiting for completion of cytogenetic analysis (Adinolfi *et al.*, 2001; Cirigliano *et al.*, 2001b, 2003). This policy is now followed in other selected genetic units, especially if ultrasound examination confirms the presence of affected fetuses.

The STRs selected on chromosomes X, Y and 21 were informative in 100% of samples; the markers on chromosomes 13 and 18 allowed correct assessment of chromosome copy number in 99.6% of cases; only 77 normal samples were found homozygous for all the selected markers, but with the inclusion of more STRs for these chromosomes, this number declined to 0.075%.

Molecular detection of fetal sex by amplification of the AMXY sequence was in agreement with cytogenetic analysis in all but two cases. Only in two male fetuses was the Y sequence of the gene found to be deleted, thus generating a pattern that was indistinguishable from a normal female. In both cases the X22 pseudoautosomal marker was heterozygous and thus compatible with the diagnosis of normal female fetuses. It has to be stressed that X22 was the only marker available to detect two sex chromosomes in these samples; thus, homozygosity of this STR with concomitant AMY deletion could have resulted in misdiagnosis of Turner's syndrome. Accordingly, soon after the detection of the first AMY deletion case, in both laboratories QF-PCR diagnoses of 45,X in the absence of suggestive ultrasound evidence, were further investigated using the SRY marker. Our results confirm a previous finding (Santos *et al.*, 1998) that deletion of the amelogenin gene on the Y chromosome is a very rare event in our population. However, a higher frequency of this polymorphism (up to 3.6% of males) has recently been reported in Indian and Malay ethnic groups (Chang *et al.*, 2003). To screen these populations it is strongly advisable to include different Y chromosome sequences, such as SRY, in multiplex assays in order to increase the reliability of sex detection.

Either the X- or the Y-specific products of the amelogenin gene were found duplicated in five male fetuses with normal karyotype. Also in these cases multiplex QF-PCR of the pseudoautosomal X22 marker together with several X-linked STRs allowed the correct diagnosis. The homologous region of the sex chromosomes containing the AMXY gene allows generation of chromosome-specific PCR products and is widely used for sex determination of DNA samples. We show that, beside deletions of the Y sequence, duplications of both the X and the Y specific products are also possible and, although very rare, these can only be detected by QF-PCR analysis in male subjects.

The comparison of fluorescent PCR products of the X-linked HPRT and D21S1411 markers allowed the assessment of X chromosome copy number in female fetuses homozygous for up to four X chromosome STRs (Cirigliano *et al.*, 2002). It was also possible to confirm that multiplex of these two sequences was of diagnostic value for the prenatal detection of fetuses with Turner syndrome independently from allele frequencies and heterozygosity of the employed markers (Cirigliano *et al.*, 2002).

As shown in previous investigations (Cirigliano *et al.*, 1999, 2001a,b), prenatal diagnoses of Turner mosaicisms by QF-PCR were hampered by the difficulty of assessing the type and ratios of the various subpopulations of cells. However, in the majority of cases, the QF-PCR tests allowed at least to deduce the diagnosis of mosaicisms by the detection of abnormal fluorescent peak ratios for the employed X chromosome STRs. In some mosaic fetuses, the ratios between 46,XX/45,X or 45,X/47,XXX or even 46,XX/ 45,X /47,XXX cells may be such as to produce fluorescent peak ratios similar to those of normal 46,XX or 47,XXX fetuses, as previously observed (Cirigliano *et al.*, 1999, 2001a,b). In some cases of X chromosome mosaicism, discrepancies between the QF-PCR and cytogenetics results regarding the percentages of abnormal cell subpopulations are probably due to the different *in vitro* cell growth of normal (46,XX or 46,XY) and 45,X cells (Cirigliano *et al.*, 2001a) with the aneuploid cell line generally growing faster than the normal. For the same reason, in this series, only the comparison between QF-PCR result on direct sample and karyotype of cultured cells allowed correct detection of three mosaic cases.

The difficulty of detecting some sex chromosome abnormalities must be evaluated in the context of the present view about the advantages of performing targeted prenatal tests for selected chromosome disorders (Adinolfi and Sherlock, 2001; Mann *et al.*, 2001; Donaghue *et al.*, 2003; Brun *et al.*, 2004). In some genetic Centres, sex chromosome-specific QF-PCR assays are not routinely performed for all cases (Mann *et al.*, 2001); only samples retrieved from fetuses suspected by ultrasound of having such chromosome disorders are tested (Donaghue *et al.*, 2003). However, sex chromosome aneuploidies such as 47,XXY, 47,XXX, and 47,XYY are generally not referred for abnormal ultrasound, as well as, in the present study, one 45,X fetus. Routine inclusion of primers for QF-PCR diagnosis of sex chromosome aneuploidies allows detection of all such cases before completion of fetal karyotype, being of great help for pregnancy management and leaving more time for parents to make a decision.

QF-PCR amplification of microsatellites has proven to be a very safe procedure for rapid and simple prenatal diagnosis. In clinical settings it has the added value to allow testing samples suspected of being contaminated by maternal cells. In this series, 155 normal male and female fetuses together with two aneuploidies were identified with great confidence, even if in the presence of heavily blood-stained amniotic fluid samples. Maternal cell contamination, if present, was always detected as characteristic QF-PCR patterns with extra peaks or skewed ratios for all chromosome markers that could not be confused with triploidy or mosaicism.

Using at least two informative sequences on each chromosome, rare polymorphic STR duplications were detected in 19 samples. In these cases the trisomic pattern observed for a single STR was in contrast with the normal heterozygous profile of all the other markers on the same chromosome. While the same result could also be

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due to partial trisomies (i.e. unbalanced translocation), the analysis of both parents with the same marker allowed us to distinguish the rare inherited polymorphism in 15 out of 19 cases.

The finding of submicroscopic duplications in microsatellites should also be a warning about the risk in reporting an autosomal trisomy detected with a single informative STR. Several chromosome-specific markers (at least three different sequences) should always be included in multiplex assays to confirm the diagnosis of aneuploidy.

Samples showing normal heterozygous pattern for a single STR should also be considered carefully; mutation in primer binding site causing null alleles, even if very rare, could hamper the detection of trisomies. In our series the only false negative result was observed in the diagnosis of a Turner syndrome fetus with a normal heterozygous pattern in only one of the four employed X-linked markers. This result is highly likely to be due to a submicroscopic duplication of this sequence that caused the artefact. This diagnosis was performed during the first year of activity when only three STRs on the X and the pseudoautosomal X22 marker were analysed. The inclusion of up to six markers and the D21S1411 used as internal control to quantify the X-linked HPRT greatly improved the efficiency of the QF-PCR assay in detecting X monosomy.

In our study, somatic microsatellite instability was only observed in three cases. This is in contrast with a recent report of high frequencies of the phenomenon in prenatal samples (Mann *et al.*, 2003). In their study, 4.2% CVS samples exhibited unbalanced triallelic patterns for a single STR marker only in a proportion of cells; this was interpreted as evidence of somatic generation of a *de novo* allele. In our series this was an extremely rare pattern only observed in one out of 625 CVS (0.2%). However, it should be stressed that these mutations do not affect the QF-PCR diagnosis when several STR markers on each chromosome are employed in multiplex assays.

Compared with FISH analysis, the clinical application of QF-PCR has the same limitation of detecting selected major numerical chromosome disorders, but, as shown in the present study, with several advantages that increase its clinical utility. The assay is very sensitive so that only a very small volume of sample is needed for the analysis. The use of highly polymorphic STR markers allows the testing of samples suspected of being contaminated with maternal cells as well as the assessment of zygosity in multiple pregnancies. The use of several highly polymorphic and chromosome-specific STRs also makes it possible to detect partial trisomies. The efficiency of the assay is not influenced by the gestational age and the high rate of uninformative samples, described using FISH (Bryndorf *et al.*, 1997), has not been observed in the course of this study.

In conclusion, the present results confirm that QF-PCR is a rapid, simple and accurate prenatal diagnostic test; in the great majority of cases it has been straightforward. The inclusion of primers for chromosomes X, Y, 21, 18 and 13 allows the detection of the great majority of clinically significant chromosome abnormalities. Because of a residual risk, in developed countries, QF-PCR can be used as a preliminary tool to reduce parental anxiety and, eventually, to accelerate therapeutic interventions, but conventional cytogenetic analysis should still be performed to confirm the prenatal diagnoses of normal fetuses. The automation of the procedure easily allowed a single operator to handle up to 40 samples at the same time and the results made available on the next working day. The availability of multicapillary DNA sequencers enables high throughput of samples at very low cost, thus allowing prenatal diagnosis of common chromosome aneuploidies in countries with large populations and limited economic resources for setting up conventional cytogenetic tests (Adinolfi *et al.*, 2000).

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References

Adinolfi M and Crolla J (1994) Nonisotopic in situ hybridization. Clinical cytogenetics and gene mapping applications. Adv Hum Genet 22, 187–255.

Adinolfi M and Sherlock J (2001) Prenatal detection of chromosome disorders by QF-PCR. Lancet. 29 (358),1030–1031.

Adinolfi M, Pertl B and Sherlock J (1997) Rapid detection of aneuploidies by microsatellite and the quantitative fluorescent polymerase chain reaction. Prenat Diagn 17,1299–1311.

Adinolfi M, Sherlock J, Cirigliano V and Pertl B (2000) Prenatal screening of aneuploidies by quantitative fluorescent PCR. Community Genet 3, 50–60.

Andonova S, Vazharova R, Dimitrova V, Mazneikova V, Toncheva D and Kremensky I (2004) Introduction of the QF-PCR analysis for the purposes of prenatal diagnosis in Bulgaria, estimation of applicability of 6 STR markers on chromosomes 21 and 18. Prenat Diagn 24,202–208.

Bili C, Divane A, Apessos A, Konstantinos T, Apostolos A, Ioannis B, Periklis T and Florentin L (2002) Prenatal diagnosis of common aneuploidies using quantitative fluorescent PCR. Prenat Diagn 22,360–365.

Bryndorf T, Christensen B, Vad M et al. (1997) Prenatal detection of chromosome aneuploidies by fluorescence in situ hybridization: experience with 2000 uncultured amniotic fluid samples in a prospective preclinical trial. Prenat Diagn 17,333–341.

Brun JL, Gangbo F, Wen Z, Galant K, Taine L, Maugey-Laulom B, Roux D, Mangione R, Horovitz J and Saura R (2004) Prenatal diagnosis and management of sex chromosome aneuploidy: a report on 98 cases. Prenat Diagn 24,213–218.

Chang YM, Burgoyne LA and Both K (2003) Higher failures of amelogenin sex test in an Indian population group. J Forensic Sci 48,1309–1313.

Cirigliano V, Sherlock J, Conway G, Quilter C, Rodeck C and Adinolfi M (1999) Rapid detection of chromosomes X and Y aneuploidies by quantitative fluorescent PCR. Prenat Diagn 19,1099–1103.

Cirigliano V, Lewin P, Szpiro-Tapies S, Fuster C and Adinolfi M (2001a) Assessment of new markers for the rapid prenatal detection of aneuploidies by quantitative fluorescent PCR (QF-PCR). Ann Hum Genet 65, 421–427.

Cirigliano V, Ejarque M, Canadas MP, Lloveras E, Plaja A, Perez MM, Fuster C and Egozcue J (2001b) Clinical application of multiplex quantitative fluorescent polymerase chain reaction (QF-PCR) for the rapid prenatal detection of common chromosome aneuploidies. Mol Hum Reprod 7, 1001–1006.

Cirigliano V, Ejarque M, Fuster C and Adinolfi M (2002) X chromosome dosage by quantitative fluorescent PCR and rapid prenatal diagnosis of sex chromosome aneuploidies. Mol Hum Reprod 8,1042–1045.

Cirigliano V, Canadas P, Plaja A, Ordonez E, Mediano C, Sanchez A and Farran I (2003) Rapid prenatal diagnosis of aneuploidies and zygosity in multiple pregnancies by amniocentesis with single insertion of the needle and quantitative fluorescent PCR. Prenat Diagn 23,629–633.

Donaghue C, Roberts A, Mann K and Ogilvie CM (2003) Development and targeted application of a rapid QF-PCR test for sex chromosome imbalance. Prenat Diagn 23,201–210.

Eiben B, Trawicki W, Hammans W, Goebel R and Epplen JT (1998) A prospective comparative study on fluorescence in situ hybridization (FISH) of uncultured amniocytes and standard karyotype analysis. Prenat Diagn 18, 901–906.

Evans MI, Henry GP, Miller WA, Bui TH, Snidjers RJ, Wapner RJ, Miny P, Johnson MP, Peakman D, Johnson A et al. (1999) International, collaborative assessment of 146,000 prenatal karyotypes: expected limitations if only chromosome-specific probes and fluorescent in-situ hybridization are used. Hum Reprod 14,1213–1216.

Levett LJ, Liddle S and Meredith R (2001) A large-scale evaluation of amnio-PCR for the rapid prenatal diagnosis of fetal trisomy. Ultrasound Obstet Gynecol 17,115–118.

Mann K, Fox SP, Abbs SJ, Yau SC, Scriven PN, Docherty Z and Ogilvie CM (2001) Development and implementation of a new rapid aneuploidy diagnostic service within the UK National Health Service and implications for the future of prenatal diagnosis. Lancet 358,1057–1061.

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Mann K, Donaghue C and Ogilvie CM (2003) In vivo somatic microsatellite mutations identified in non-malignant human tissue. Hum Genet 114, 110–114.

Mansfield ES (1993) Diagnosis of Down Syndrome and other aneuploidies using quantitative polymerase chain reaction and small tandem repeat polymorphisms. Hum Mol Genet 2,43–50.

Marteau TM, Cook R, Kidd J, Michie S, Johnston M, Slack J and Shaw RW (1992) The psychological effects of false-positive results in prenatal screening for fetal abnormality: a prospective study. Prenat Diagn 12, 205–214.

Pergament E, Chen PX, Thangavelu M and Fiddler M (2000) The clinical application of interphase FISH in prenatal diagnosis. Prenat Diagn 20, 215–220.

Pertl B, Yau SC, Sherlock J, Davies AF, Mathew CG and Adinolfi M (1994) Rapid molecular method for prenatal detection of Down's syndrome. Lancet 343,1197–1198.

Pertl B, Weitgasser U, Kopp S, Kroisel PM, Sherlock J and Adinolfi M (1996) Rapid detection of trisomies 21 and 18 and sexing by quantitative fluorescent multiplex PCR. Hum Genet 98,55–59.

Pertl B, Kopp S, Kroisel PM, Tului L, Brambati B and Adinolfi M (1999a) Rapid detection of chromosome aneuploidies by quantitative fluorescence PCR: first application on 247 chorionic villus samples. J Med Genet 36, 300–303.

Pertl B, Pieber D, Lercher-Hartlieb A, Orescovic I, Haeusler M, Winter R, Kroisel P and Adinolfi M (1999b) Rapid prenatal diagnosis of aneuploidy by quantitative fluorescent PCR on fetal samples from mothers at high risk for chromosome disorders. Mol Hum Reprod 5,1176–1179.

Santos FR, Pandya A and Tyler-Smith C (1998) Reliability of DNA-based sex tests. Nat Genet 18,103.

Schmidt W, Jenderny J, Hecher K, Hackeloer B, Kerber S, Kochhan L and Held KR (2000) Detection of aneuploidy in chromosomes X, Y, 13, 18 and 21 by QF-PCR in 662 selected pregnancies at risk. Mol Hum Reprod 6,855–860.

Sherlock J, Cirigliano V, Petrou M, Tutschek B and Adinolfi M (1998) Assessment of diagnostic quantitative fluorescent multiplex polymerase chain reaction assays performed on single cells. Ann Hum Genet 62,9–23.

Sjogren B and Uddenberg N (1990) Prenatal diagnosis for psychological reasons: comparison with other indications, advanced maternal age and known genetic risk. Prenat Diagn 10,111–120.

Tepperberg J, Pettenati MJ, Rao PN, Lese CM, Rita D, Wyandt H, Gersen S, White B and Schoonmaker MM (2001) Prenatal diagnosis using interphase fluorescence in situ hybridization (FISH): 2-year multi-center retrospective study and review of the literature. Prenat Diagn 21,293–301.

Ward BE, Gersen SL, Carelli MP, McGuire NM, Dackowski WR, Weinstein M, Sandlin C, Warren R and Klinger KW (1993) Rapid prenatal diagnosis of chromosomal aneuploidies by fluorescence in situ hybridization: clinical experience with 4,500 specimens. Am J Hum Genet 52,854–865.

Voglino G, Marongiu A, Massobrio M, Campogrande M and Todros T (2002) Rapid prenatal diagnosis of aneuploidies. Lancet 359,442.

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