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Rapid prenatal diagnosis of common chromosome aneuploidies by QF-PCR, results of 9 years of clinical experience†

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Background Despite being deliberately targeted to common chromosome aneuploidies, the rapid quantitative fluorescent polymerase chain reaction (QF-PCR) tests can detect the majority of chromosome abnormalities in prenatal diagnosis. The main advantages of this assay are low cost, speed and automation allowing large-scale application.

Methods We developed a QF-PCR test that was applied on 43 000 clinical samples reporting results in 24 h. Most common indications were biochemical risk (32%) and advanced maternal age (30%). Samples were also tested by cytogenetic analysis and the results compared.

Results Aneuploidies involving chromosomes 21, 18, 13, X and Y were detected with 100% specificity. Several cases of partial trisomies and mosaicism were also identified. Overall 95% of clinically relevant abnormalities were readily detected and termination of affected pregnancies could be performed without waiting for the cytogenetic results.

Conclusions Our study supports the possibility of reducing the load of prenatal cytogenetic tests if the pregnancies are carefully monitored by non-invasive screening. In case of abnormal QF-PCR results, medical action can be taken within few hours from sampling. In cases of negative QF-PCR results, cytogenetic analyses might only be performed for fetuses with ultrasound abnormalities. In countries where large-scale cytogenetic tests are not available, QF-PCR may be used as the only prenatal diagnostic procedure.

KEY WORDS: QF-PCR; STR; aneuploidy

INTRODUCTION

Over the last four decades prenatal diagnoses of chromosome abnormalities have been carried out by cytogenetic analysis of amniotic, chorionic or fetal blood cells that, after being cultured *in vitro* for several days, are examined in metaphase using appropriate staining procedures. Chromosome analyses allow accurate detection of both numerical and structural abnormalities in all the chromosomes. The main disadvantage is the long time needed for culturing fetal cells; the interval between the collection of the samples and the final report (around 10–14 days) can be a very anxious time for the parents, particularly if non-invasive screening tests (biochemical and/or ultrasound), performed during the first or second trimester of pregnancy, have suggested an increased risk of chromosome disorders (Sjogren and Uddenberg, 1990; Marteau *et al*., 1992).

The quantitative fluorescent polymerase chain reaction (QF-PCR) assay has been developed in the last 15 years to detect major numerical chromosome disorders in a few hours after the collection of the samples (Mansfield, 1993; Pertl *et al*., 1994, 1996, 1997, 1999a,b; Adinolfi *et al*., 1995, 1997; Sherlock *et al*., 1998; Verma *et al*., 1998; Cirigliano *et al*., 1999, 2001b, 2004; Adinolfi and Sherlock, 2001a).

Trisomies are identified by detecting three doses of chromosome-specific short tandem repeats (STRs); sexing can be performed by simultaneous amplification of the Y chromosome-specific SRY sequence and the modified non-polymorphic Amelogenin (AMXY), which produces chromosome X- and Y-specific products (Figure 1).

The clinical utility of the QF-PCR assay has repeatedly been confirmed by its high sensitivity and specificity in detecting major chromosome abnormalities (Pertl *et al*., 1999a,b; Schmidt *et al*., 2000; Adinolfi and Sherlock., 2001a; Cirigliano *et al*., 2001a, 2004, 2006;

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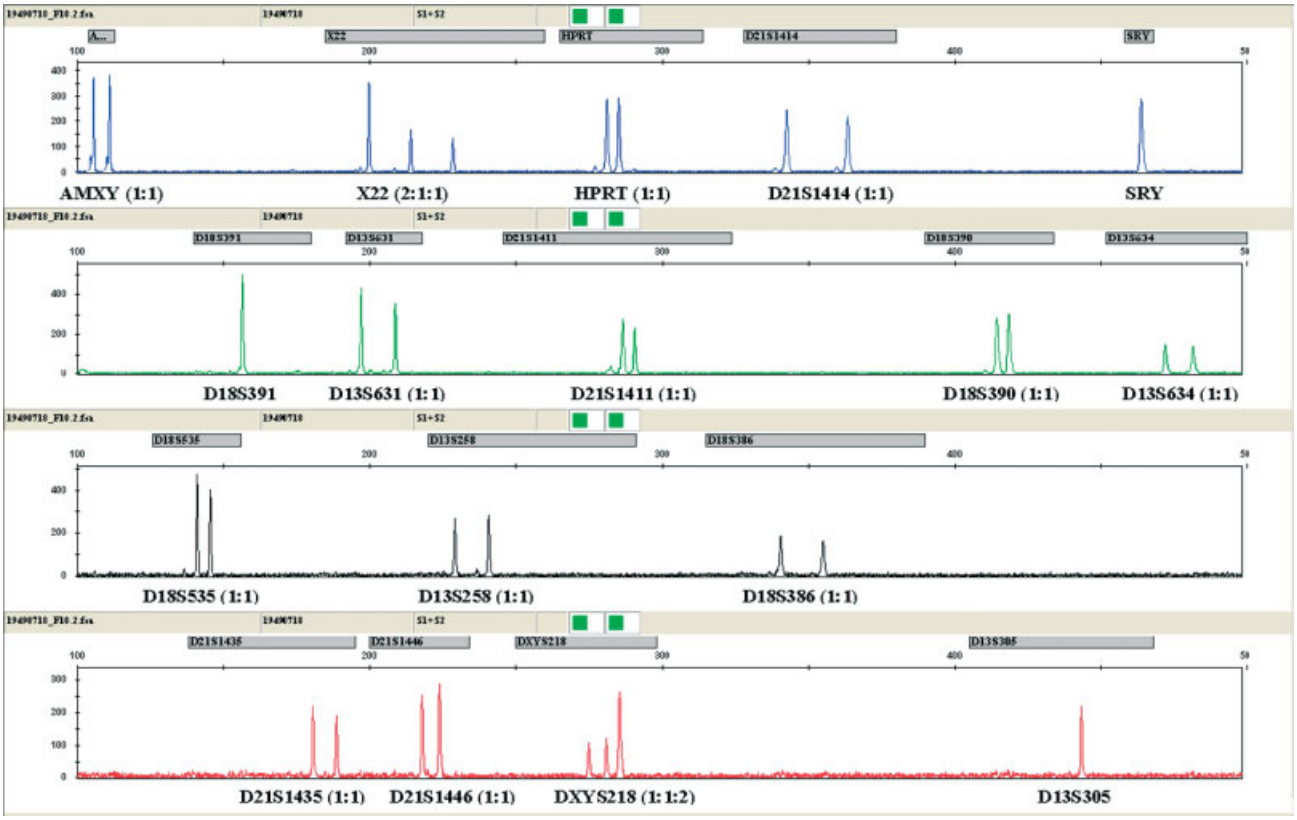

Figure 1—Electrophoretogram showing the QF-PCR detection of a 48,XXYY fetus. Products amplified using the M1 and M2 assays are collected and analysed in a single injection. The same copy number for the X and Y chromosomes is detected by the 1 : 1 ratio observed between the X- and Y-specific products of the AMXY. Male sex is confirmed by the presence of the SRY. Two copies of the X chromosomes are revealed by the heterozygous pattern (1 : 1) of the HPRT marker. Both pseudoautosomal STRs (X22 and DXYS218) are highly informative for the presence of four sex chromosomes because of the presence of two minor alleles (1 : 1) from the X chromosomes together with a double peak (from the Y chromosomes). Normal chromosome copy number is detected for the autosomes, with four informative markers on chromosome 21 and 3 on each chromosome 13 and 18

Mann *et al*., 2001, 2004). One of the advantages of QF-PCR is the automation of the procedure that allows high throughput of samples at a very low cost (Adinolfi *et al*., 1997, 2000; Cirigliano *et al*., 2005, 2006).

QF-PCR tests are now performed in several prenatal centres in Europe for the detection of major numerical abnormalities affecting chromosomes 21, 18, 13, X and Y, with results provided in 24 h (Shmidt *et al*., 2000; Mann *et al*., 2001; Bili *et al*., 2002; Voglino *et al*., 2002; Cirigliano *et al*., 2003, 2004, 2006; Andonova *et al*., 2004; El Mouatassim *et al*., 2004; Onay *et al*., 2008; Putzova *et al*., 2008).

Here we report the results of screening 43 000 consecutive fetal samples collected over 9 years in two different genetic centres using both QF-PCR and conventional cytogenetic analysis. Overall 99.6% of fetuses with normal karyotype was correctly identified by QF-PCR without false positive results. In the absence of high levels of contaminating maternal cells, all fetuses with trisomy 21, 18, 13, triploidy and all, but one case, of X and Y chromosome aneuploidies were correctly diagnosed. In the great majority of cases parents could be informed about the results within 24 h from the collection of the sample. The accuracy of the QF-PCR tests allowed early termination of affected pregnancies without waiting for the results of conventional cytogenetic tests.

MATERIALS AND METHODS

Samples were collected between February 1999 and March 2008 in two different centres (General Lab, Barcelona, Spain and Promea S.p.A., Turin, Italy) offering rapid QF-PCR service to other cytogenetic laboratories, private clinics and public hospitals, thus receiving several samples from high-risk pregnancies nationwide on a daily basis. The majority of prenatal samples were amniotic fluids (37 544) collected between 12 and 31 weeks of gestation and CVS (4687) collected between 11 and 13 weeks of gestation; 178 fetal blood samples and 591 fetal tissues from aborted fetuses were also investigated. In 191 prenatal cases, QF-PCR was not requested at the time of collecting the samples but it was performed afterwards on cytogenetic cultures. These included slow growing 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 confirm the fetal origin of the predominant cell population.

The most common indications for an invasive procedure were: raised risk of chromosome disorders for advanced maternal age (30%), biochemical screening tests performed on maternal serum (32%), 6% of these

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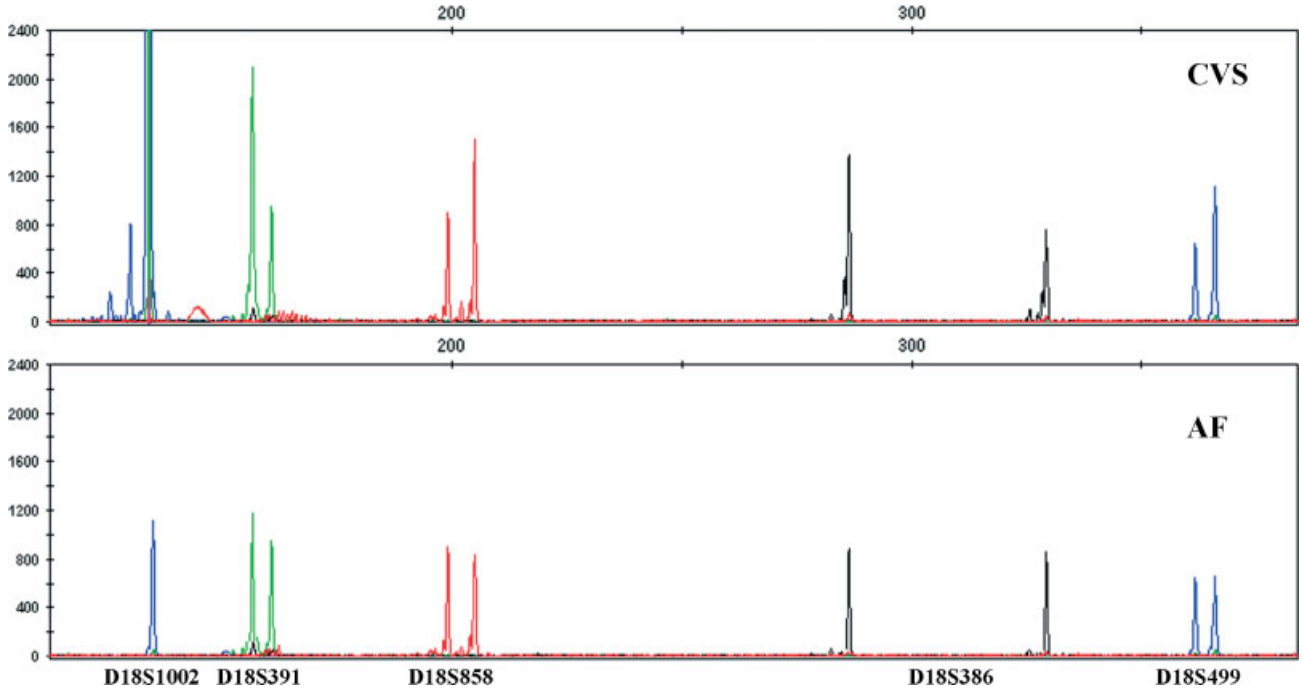

Figure 2—QF-PCR assessment of confined placental mosaicism. Trisomy 18 is detected with chromosome-specific multiplex in single CVS frond (top). All informative STRs show trisomic diallelic patterns (2 : 1) which are compatible with postzygotic or meiosis II non-disjunction. The analysis of the same markers in amniotic fluid (AF) shows the presence of a normal fetus with a mitotic T18 confined to the placenta

cases were also associated with an increased nuchal translucency; parental anxiety generated 22% of samples; abnormal ultrasound findings were present in 7% of fetuses. 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- to 1-ml amniotic fluids or cell cultures, 5 µl of fetal bloods, 200-µl buccal washes, a small villous fragment or 1- to 2-mm tissue sections using a Chelex based procedure (Instagene Matrix, Bio-Rad Laboratories, CA), as previously described (Cirigliano *et al*., 2001a). QF-PCR assays were developed in both Centres and improved over the years by adding new markers for specific chromosomal regions of interest (Cirigliano *et al*., 2001b, 2002, 2003, 2004, 2006); selected markers are listed in Table 1. Since 2002, the D21S1411 was used to quantify the X-linked HPRT and thus perform prenatal diagnosis of X monosomy (Cirigliano *et al*., 2002).

The use of the non-polymorphic sequence of the Amelogenin (AMXY) gene was complemented by the Y chromosome SRY to detect fetal sex. All selected STRs were stable tri-, tetra- and penta-nucleotides, suitable for multiplex PCR because of their very low frequency of artefacts produced during amplification. The STRs' locations along the examined chromosomes were also taken into account in order to increase the possibility of detecting partial trisomies (Table 1). All forward primers were labelled with fluorescent molecules allowing accurate sizing and quantification of the final products. As shown in Table 1, primers producing amplicons of similar sizes were labelled with different fluorochromes in order to be amplified 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 for multiplex QF-PCR reactions in order to simplify sample handling and data analysis (Cirigliano *et al*., 2001a,b, 2004, 2006; Voglino *et al*., 2002).

Since May 2006, the commercially available Aneufast QF-PCR Kit (Molgentix, Spain) that includes the previously selected markers in six multiplex assays was used in both centres.

Aneuploidy screenings were performed amplifying four STRs on chromosomes 21, 18, 13, two pseudoautosomal, one X linked, as well as the AMXY and SRY; markers were distributed in two multiplex QF-PCR assays (M1 and M2) in order to reduce the risk of sample mishandling (Table 2). Following collection of the products and simultaneous electrophoretic analysis, agreement between results from the two multiplexes allows diagnosis to be performed with two independent assays on each sample.

Samples with less than two informative markers on each chromosome, were re-tested using chromosome-specific multiplex PCR assays including up to seven STRs on chromosomes 21 and 18, eight STRs on the X, and six markers on chromosome 13 (Table 2). These sets of additional markers were also used to confirm sample identity in all aneuploid cases by testing a second aliquot obtained from the original sample.

The fluorescent QF-PCR products and size standards were analysed by capillary electrophoresis on ABI 3100 Avant, ABI 3130 and 3130XL automated DNA sequencers using Genescan 3.7, GeneMapper 3.7 and 4.0 (Applied Biosystems, Foster City, CA) or Genemarker

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Table 1—Markers selected for QF-PCR detection of chromosome aneuploidies. Sequences producing amplicons of similar sizes are labelled with different fluorochromes to be analysed in the same electrophoresis. Heterozygosity is reported as that observed in our study. Markers are selected along each examined chromosomes in order to increase the likelihood of detecting partial imbalance

Marker	Label	Het.	Chromosome location	Common alleles in base pair
AMXY	6-Fam	—	Xp22.1–22.31 - Yp11.2	X 104 Y 109
SRY	6-Fam	—	Yp11.2	Y 463
X22	6-Fam	0.91	Xq28 Yq (PAR2)	189-194-199-204-209-214-219-224-226-229-234-239-242-247-253
DXYS218	PET	0.75	Xp22.32 Yp11.3 (PAR1)	266-270-274-278-282-286-290-294
HPRT	6-Fam	0.75	Xq26.1	264-268-272-276-278-280-284-288-292-296-300-313
DXS6803	VIC	0.68	Xq12-Xq21.33	106-110-114-118-120-124-128
DXS6809	VIC	0.75	Xq	238-242-246-250-252-254-258-260-262-266-268-270-274
DXS8377	NED	0.85	Xq28	213-216-219-222-225-228-238-241-244-248-252
SBMA	VIC	0.75	Xq11.2-Xq12	166-169-172-175-178-181-184-187-190-193-196-199-202-205-208-211
D21S1414	6-Fam	0.85	21q21	328-330-334-338-342-346-350-352-354-356-358-360-362-443
D21S1411	VIC	0.93	21q22.3	246-262-266-274-278-282-286-290-294-298-302-306-316-319
D21S1446	PET	0.77	21q22.3-ter	200-204-208-212-214-218-220-224-228
D21S1437	VIC	0.78	21q21.1	120-124-128-132-136-140-144-148
D21S1809	6-Fam	0.70	21q22.1	192-196-200-204-208-212-216-220-224
D21S1412	6-Fam	0.73	21q22.2	384-388-392-396-400-406-410-414-418
D21S1435	PET	0.75	21q21	142-160-164-168-172-176-180-184-188
D18S391	VIC	0.75	18pter-18p11.22	140-144-148-152-156-160-164-168
D18S390	VIC	0.75	18q22.2	398-402-406-410-414-418-422-426-430
D18S535	NED	0.82	18q12.2	126-130-134-138-142-146-148-152-156
D18S386	NED	0.89	18q22.1	319-330-334-338-342-344-350-354-358-362-366-370-372-376-380-387
D18S858	PET	0.76	18q21.1	186-190-192-196-200-204-208-212-216
D18S499	6-Fam	0.72	18q21.32-q21.33	386-392-396-400-404-408-412-416
D18S1002	6-Fam	0.80	18q11.2	118-122-130-134-138-142-146-150-154
D13S631	VIC	0.78	13q31-32	192-196-200-204-208-212-215-218
D13S634	VIC	0.85	13q14.3	460-464-466-470-474-478-482-484-486-490-496-500
D13S258	NED	0.89	13q21	232-234-236-238-240-242-244-248-265-267-269-271-273-277-279-281
D13S305	PET	0.75	13q12.1-13q14.1	426-430-434-438-442-446-450-454-458
D13S628	6-Fam	0.70	13q31-q32	436-440-444-448-452-456-460-464
D13S742	VIC	0.75	13q12.12	254-258-262-266-268-270-274

Table 2—Multiplex assays included in the Aneufast™ QF-PCR Kit. Mix 1 and 2 are used to screen all prenatal samples with four markers on chromosomes 13, 18 and 21, two pseudoautosomal X and Y and one X-linked marker. AMXY and SRY are used for sexing. Two autosomal markers and sexing sequences are present in both multiplexes, this allow obtaining results with two independent assays on each sample

M1	M2	MXY	M21	M18	M13
AMXY	SRY	SRY	D21S1411	D18S386	D13S631
D21S1414	X22	AMXY	D21S1435	D18S391	D13S634
D21S1446	DXYS218	HPRT	D21S1437*	D18S858*	D13S742*
D13S631	HPRT	SBMA*	D21S1412*	D18S499*	D13S628*
D13S305	D21S1411	DXS6803*	D21S1809*	D18S1002*	
D18S535	D21S1435	DXS6809*			
D18S391	D13S634	DXS8377*			
	D13S258				
	D18S386				
	D18S390				

If required, additional markers (*) on selected chromosomes are added with the correspondent chromosome-specific assays including two markers already present in the M1M2 assays to confirm the identity of the sample.

Software (SoftGenetics, State College, PA) as previously described (Pertl *et al*., 1996; Adinolfi *et al*., 1997; Cirigliano *et al*., 2001b, 2002). All prenatal samples were processed and reported within 24–48 h.

Conventional cytogenetic analyses were performed on all the prenatal samples, cultured and harvested according to standard procedures; depending on the specimen, the results were issued between 5 and 22 days (mean reporting time of two weeks for amniotic fluids).

RESULTS

As shown in Table 3, 41 019 cases (95%) were correctly identified as chromosomically normal by the QF-PCR

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Table 3—Results of testing 43 000 consecutive clinical samples by QF-PCR and cytogenetic analysis

Karyotype	QF-PCR	Cytogenetics
46,XX; 46,XY	41 019	41 178
47,XX +21; 47,XY+21	751	753[a]
47,XX +18; 47,XY+18	298	299[a]
47,XX+13; 47,XY+13	127	127
69,XXX; 69,XXY; 69,XYY	103	103
45,X	105	106
47,XXY	65	66[a]
47,XYY	48	48
47,XXX	36	32[b]
49,XXXXX	2	2
49,XXXXY	3	3
48,XXYY	2	2
48,XXY+21	4	4
48,XXY+18	3	3
68,XX	1	1
Mosaics	45	72
92,XXXX	—	2
Other aneuploidies	—	39
Structural balanced	—	47
Structural unbalanced	15	32
MCC[c]	352	29
Failed tests[d]	21	52
Total abnormalities	1608	1741

[a] Four aneuploid cases could not be detected by QF-PCR because of the presence of high level maternal cell contamination (MCC).
[b] Four samples diagnosed as 47,XXX by QF-PCR were shown to be 45,X/46,XX mosaic by cytogenetic analysis.
[c] Samples uninformative because of the presence of maternal cell contamination or maternal cell overgrowth in culture.
[d] QF-PCR failure occurred in samples not tested soon after collection but in cell cultures heavily contaminated by bacteria or fungal cells.

assay out of 41 178 cases diagnosed as normal by conventional cytogenetic tests (99.6%). The detection of heterozygous patterns with fluorescent peaks ratios close to 1 : 1 for at least two chromosome-specific STRs was sufficient to perform the diagnosis. No false positive results were observed. Using the full set of autosomal and sex chromosome STRs, 1608 fetuses were diagnosed as chromosomically abnormal out of 1741 abnormalities detected by conventional cytogenetic analysis (92.3% sensitivity) within 24 h from collection of the samples (Table 3). In samples referred for abnormal ultrasound findings, QF-PCR detected 95% of all clinically significant chromosome abnormalities, later assessed by cytogenetic analysis. The rapid test demonstrated 100% specificity for chromosomes 21, 18, 13, X and Y aneuploidies with positive predictive value (PPV) of 100% and negative predictive value (NPV) of 99.7%.

Fetal sexing was correctly performed in all samples but one; the only discordance was observed in an early case of normal male with deletion of the Y-specific amelogenin (AMLX) sequence that was interpreted as the pattern of a female fetus. This prompted the inclusion in all cases of the SRY sequence to confirm the AMXY results that allowed the correct sexing of four more fetuses with such polymorphism.

As shown in Table 3, the rapid prenatal detection of fetuses with triploidies (69,XXX, 69,XXY and 69,XYY), double trisomies (48,XXY+21 and 48,XXY +18) and different aneuploidies involving the X and Y chromosomes was also highly successful.

One case of Turner syndrome (out of 106) was not detected during the early phase of the QF-PCR testing, however none went missed after adding all sex chromosomes specific STRs listed in Table 1. The inclusion of D21S1411 as internal control to quantify the HPRT also made possible to detect one rare female fetus homozygous for four X-linked markers and one normal fetus homozygous for five markers on chromosome 21.

QF-PCR was effective in detecting 45 out of 72 fetuses with chromosome mosaicism (Table 3). The presence of two different cell lines in the prenatal samples was detected in all cases of 45,X/46,XY mosaicism (Figure 2). In four of these cases, the presence of a cell line with the Y chromosome could only be demonstrated by the QF-PCR detection, in the uncultured samples, of a small Y-specific fluorescent PCR product of the AMXY; in these cases cytogenetic analysis resulted in pure 45,X. Mosaicism 45,X/46,XX could be suspected by QF-PCR on the evidence of unbalanced allele ratios for the employed X chromosome markers, when the aneuploid cell line was present in at least 20% of the cells. Chromosomes 21, 18 and 13 mosaics could be diagnosed in several cases as small extra alleles for at least two chromosome-specific STRs. In agreement with previous studies (Cirigliano *et al*., 1999, 2001a,b; Schmidt *et al*., 2000; Donaghue *et al*., 2005), low levels of chromosome mosaicisms could not be detected by QF-PCR in the remaining cases.

QF-PCR amplification of STR markers also allowed discriminating meiotic and mitotic non-disjunction in CVS samples; confined placental mosaicism could be suspected in eight CVS by QF-PCR because of trisomic diallelic patterns observed for all informative markers in single tissue fronds (Figure 2); six true mosaic fetuses were identified by QF-PCR because of the trisomic triallelic STR patterns confirming the meiotic origin of the extra chromosome.

DNA extraction and QF-PCR amplification were successful in all but 21 samples, these cases had not been analysed by QF-PCR soon after the collection since the molecular tests were only requested after cell culture failures. Conventional cytogenetic analysis was not achieved in a total of 81 prenatal cases because of cell culture failures (52 samples) or maternal cell overgrowth (29 cases). In 48 of these cases, QF-PCR tests were successful and gave normal results, thus a second invasive procedure could be avoided. In the present series QF-PCR was attempted even in samples suspected of being contaminated with maternal cells, in a high proportion of these cases (~85%) QF-PCR analysis was possible including the diagnoses of normal female fetuses after testing maternal buccal cells with the same markers.

In 352 samples, STR analysis showed evidence of high levels maternal cell contamination and no result could be obtained other than fetal sex, these included four cases of chromosome abnormalities that could not be detected.

Of course, not being set up to detect all chromosome abnormalities, 39 aneuploidies involving chromosomes

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5, 8, 14, 15, 16, 22, and small extra chromosome markers were missed, but 15 out of 32 cases of partial imbalances for the tested chromosomes could be detected. As expected, none of the 47 balanced translocations were detected by the molecular test.

Submicroscopic polymorphic duplications of microsatellites were observed in 59 samples as clear trisomic triallelic or diallelic patterns for one chromosome-specific STR. Duplications were observed in D21S1446, D21S1411, D21S1414, D13S631, D13S634, D18S386, DXYS218 and X22. In most cases, the parental origin of the abnormality could be assessed by testing both parents with the same marker. Nine more normal samples (eight CVSs, one amniotic fluid) produced unbalanced triallelic patterns for a single marker consistent with somatic microsatellite instability. However, all these samples were further analysed using other informative chromosome-specific STRs and this allowed performing the correct prenatal diagnoses.

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

DISCUSSION

Over the last years non-invasive screenings for the identification of pregnancies at high risk of chromosomal abnormality have significantly improved (Wald, 1995; Wald and Hackshaw, 2000; Snijders *et al*., 1998). Especially with the advent of combined ultrasound and biochemical tests it is now possible to ascertain risk categories from the first trimester of pregnancy (Nicolaides *et al*., 2000, 2005; Cicero *et al*., 2003; Canick *et al*., 2006; Schiott *et al*., 2006). This is a significant advance in pregnancy management as parents can be rapidly reassured that an invasive procedure is not required or affected fetuses can be identified earlier and termination of pregnancy may be carried out using less-traumatic procedures. We have developed a rapid QF-PCR diagnostic test that has been applied to screen high-risk pregnancies before completion of fetal karyotype.

The analysis of this large series of prenatal diagnoses (43 000 consecutive clinical specimens) performed by both the QF-PCR assay and conventional cytogenetics, allowed us to assess the advantages and limitations of applying this molecular technique soon after collection of the samples.

The overall results showed that the QF-PCR is a rapid, simple and accurate diagnostic test. In 41 019 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 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 and CVS correctly diagnosed 100% of trisomies for chromosomes 21, 18, 13, triploidies, double trisomies (48,XXY,+21; 48,XXY,+18), X and Y pentasomies (49,XXXXX; 49,XXXXY) and non-mosaic aneuploidies involving both chromosomes X and Y without false negative results.

A total of 1608 aneuploidies have readily been detected by QF-PCR in the present study; this accounts for 92.3% of fetuses with abnormal karyotypes diagnosed by conventional cytogenetic tests. The sensitivity of the molecular assay increased to 95% if only clinically significant cytogenetic abnormalities are considered. Of course, as the tests were not set up to investigate all chromosome disorders, aneuploidies affecting other chromosomes were missed. As expected, unbalanced Robertsonian translocations, resulting in trisomies 21 or 13, could not be distinguished from the corresponding free trisomies.

No false positive results were observed (100% PPV) and, in several cases, the great efficiency and reliability of QF-PCR allowed early termination of affected fetuses without further waiting for the completion of cytogenetic analysis (Cirigliano *et al*., 2001a, 2002, 2004, 2005). This policy is now followed in other selected genetic units especially if ultrasound examinations confirm the presence of affected fetuses (El Mouatassim *et al*., 2004; Mann *et al*., 2004; Nicolini *et al*., 2004; Quaife *et al*., 2004).

In the course of this study, QF-PCR proved to be efficient also in detecting fetal zygosity in all cases of multiple pregnancies analysed. This can avoid repeating amniocentesis in cases of dizygotic twins of the same sex with uncertain chorionicity (Chen *et al*., 2000; Cirigliano *et al*., 2003, 2004).

The QF-PCR results could not be issued for about 1% of samples because of the presence of maternal cell contamination. Most of these specimens were heavily blood-stained amniotic fluids. QF-PCR amplification of highly polymorphic STRs of a sample heavily contaminated by maternal cells is expected to produce a characteristic pattern with extra alleles or skewed ratios between peaks for all chromosomes. These patterns are not usually compatible with a normal, trisomic or with a triploid result, so these samples can be safely tested without any risk of misdiagnosis. In the presence of low-level maternal cell contamination, the ratio between fetal STR peaks is not significantly altered; in these cases, diagnosis can be performed without great difficulties, also in the presence of female fetuses, by testing maternal DNA with the same markers.

For the same reason QF-PCR has also proven to be very helpful in testing slow growing CVS cultures resulting in normal female karyotypes along with a maternal sample to confirm the fetal origin of the analysed cells.

Molecular detection of fetal sex by amplification of the AMXY gene was found to be in agreement with cytogenetic analysis in all but five cases where the Y sequence of this gene was deleted. Accordingly, soon after the detection of the first AMY deletion case, the QF-PCR assays were further improved by including the SRY and a second pseudoautosomal marker. Our results confirm the previous finding (Santos *et al*., 1998), that deletion of the Amelogenin gene on the Y chromosome

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is a very rare event in our population but it has also been observed in a few forensic cases (Michael and Brauner, 2004; Shadrach *et al*., 2004). 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). Thus, in order to increase the reliability of sex detection, it is strongly advisable to include different Y chromosome sequences, such as SRY, to screen these populations for fetal aneuploidies by QF-PCR (Cirigliano *et al*., 2006).

As expected, the QF-PCR detection of mosaicisms was hampered by occasional difficulties, some of them in common with other prenatal diagnostic tests. First of all, low levels of mosaicism (less than 10%) may not be detected by the molecular tests. Sensitivity of QF-PCR in detecting the presence of two or more cell lines in mosaic cases, greatly depends on cell proportions and the chromosome involved. For instance, 46,XX/45,X mosaics can be recognized by the unbalanced allelic ratios of the employed X chromosome markers in cases where aneuploidy is present in at least 20% of cells. The difficulty of assessing the type and ratios of the various sub-populations of cells is evident in some mosaic fetuses where 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 (Cirigliano *et al*., 1999, 2001a,b, 2004; Schmidt *et al*., 2000). Nevertheless, Y derived sequences in 45,X/46,XY as well as extra autosomal alleles in mosaic trisomies are well detectable even in samples with only 5% of abnormal cells (Cirigliano *et al*., 1999, 2001b, 2004, Donaghue *et al*., 2005). In some cases of X chromosome mosaicism, discrepancies between the QF-PCR and cytogenetics results regarding the percentages of abnormal cell sub-populations are likely due to the different *in vitro* cell growth rate of normal (46,XX or 46,XY) and 45,X cells, with the aneuploid cell line generally growing faster than the normal (Cirigliano *et al*., 2001b, 2004). However, the QF-PCR tests allowed deducing the presence of more than one cell line in about 50% of mosaic cases diagnosed by cytogenetic analysis. The difficulty of detecting some sex chromosome abnormalities must be evaluated in the contest of the present view about the advantages of performing prenatal test for selected chromosome disorders (Adinolfi *et al*., 2001a; Mann *et al*., 2001, 2008; 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 as a result of abnormal ultrasound, as well as, in the present study, four 45,X fetuses. We strongly believe that primers for QF-PCR diagnosis of sex chromosome aneuploidies should routinely be included; the early detection of all such cases would leave more time to the parents for appropriate genetic counselling (Cirigliano *et al*., 2002, 2004, 2005, 2006).

Polymorphic STR duplications were observed in 59 samples. In 58 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 due to partial trisomies (i.e. unbalanced translocation), the analysis of both parents with the same marker allowed to distinguish the rare inherited polymorphism in the majority of cases. In our series the only false negative result was observed in the early diagnosis of a 45,X fetus with a normal heterozygous pattern in only one out of four markers used at that time. 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. However, the efficiency of the QF-PCR assay in detecting X monosomy was greatly improved, and no more cases were missed, since the inclusion of all the markers listed in Table 1 together with the D21S1411 used as internal control to quantify the HPRT (Cirigliano *et al*., 2002).

The selected markers also allowed the detection of partial chromosome imbalance in 15 out of 32 cases diagnosed by cytogenetic analysis; in such cases the diagnosis was based on the trisomic pattern observed for at least two markers.

In our study, somatic microsatellite instability was only observed in nine cases. This is in contrast with a report about high frequencies of such phenomenon in CVSs (Mann *et al*., 2003). In their study, 4.2% of 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 0.2% of CVSs. 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. Rare discrepancies between QF-PCR and karyotype results have occasionally been reported; in many instances, these were limited to testing single chorionic samples and were due to mosaicisms which are found with significant frequencies in this tissue (Allen *et al*., 2006; Waters *et al*., 2007). In order to avoid this problem, mesoderm enriched cells from digested CVS samples, prepared for culture set up, should be used together with a careful evaluation of the STR patterns obtained in trisomic cases. In the course of this study, it has been possible to identify confined placental trisomies (of postzygotic/mitotic origin) as originating trisomic diallelic patterns for all the informative markers on the involved chromosome. In trisomic fetuses, the detection of trisomic triallelic patterns in at least one STR was of great diagnostic value to discriminate the meiotic origin of the extra chromosome.

Large-scale QF-PCR tests, performed in other diagnostic centres are basically in agreement with our investigations, thus confirming that although deliberately targeted to the analysis of selected disorders affecting three autosomes and the sex chromosomes, QF-PCR can detect the great majority of chromosome abnormalities

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in prenatal samples (Pertl *et al*., 1996, 1999a,b; Schmidt *et al*., 2000; Levett *et al*., 2001; Bili *et al*., 2002; Mann *et al*., 2004; Brown *et al*., 2006; Ochshorn *et al*., 2006; Vrbicka *et al*., 2006; Kagan *et al*.,2007).

A recent review of our data suggested that the rapid QF-PCR tests may help in reducing the need of conventional cytogenetic analysis in prenatal diagnosis detecting 95% of chromosome abnormalities with potential risk for the fetus within 24 h from sampling (Cirigliano *et al*., 2005). In fetuses with abnormal ultrasound findings, the molecular assay detected 95% of all abnormalities diagnosed by cytogenetic analysis, clearly indicating that this is the particular risk category where it is possible to find chromosomal abnormalities that the molecular assay is not designed to detect (Cirigliano *et al*., 2005). These observations have recently been confirmed by retrospective analyses of large series of CVSs (Chitty *et al*., 2006) and amniotic fluids (Kagan *et al*., 2007) confirming fetal ultrasound as the main indication to decide if QF-PCR should be followed by a full fetal karyotype.

In 2004, the UK National Screening Committee suggested that rapid diagnostic tests, such as QF-PCR, should be offered to all women undergoing an invasive diagnostic procedure. Since then, the issue of whether or not to karyotype all pregnancies has also been considered, the main reasons being the financial savings to the United Kingdom National Health Service resulting from the only use of QF-PCR and a reduction of unnecessary stress caused by the incidental detection of chromosome abnormalities of little or unknown clinical significance in woman referred for increased risk of Down syndrome (Grimshaw *et al*., 2003; Ogilvie *et al*., 2005).

However, concern has been expressed that replacement of conventional cytogenetic investigations would result in a substantial number of infants affected by preventable handicaps and vivid discussion is in place between involved professional groups (Wolstenholme, 1998; Adinolfi *et al*., 2001a; Mann *et al*., 2001; Grimshaw *et al*., 2003; Leung *et al*., 2003; Ogilvie, 2003; Ogilvie *et al*., 2005; Wenstrom, 2003; Nicolini *et al*., 2004; Caine *et al*., 2005; Cirigliano *et al*., 2005).

In our view, a careful evaluation of ultrasound and biochemical tests should reduce the need to perform invasive procedures. When amniocentesis or CVS is required, the QF-PCR assay could reduce the need to perform conventional cytogenetic analyses for most prenatal samples. For example, in Sweden, following genetic counselling and in absence of ultrasound markers, the parents are offered the alternative to either choose the rapid QF-PCR test or the conventional cytogenetic analysis. Surprisingly, over 70% of women choose the option of only having the rapid QF-PCR test instead of the full cytogenetic analysis (Bui, 2007).

The use of QF-PCR approach could be of great help in countries with very large populations, for example China or India, where prenatal detection of chromosome disorders by cytogenetic analyses are still hampered by shortage of funds and specialized technicians; in these cases the QF-PCR test may be considered as the only viable option for large-scale prenatal diagnosis (Adinolfi *et al*., 2000; Cirigliano *et al*., 2006).

New approaches need to be explored and are in continuous development. It is expected that, in the near future prenatal diagnoses may be efficiently performed using new improved non-invasive methods that would probably include the detection of fetal DNA/RNA in maternal plasma (Spencer *et al*., 2003; Chiu and Lo, 2006; Lo *et al*., 2007; Lo and Chiu, 2008) or the detection of fetal cells in endocervical samples (Adinolfi *et al*., 1993; Adinolfi and Sherlock, 2001b).

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