Prenatal diagnosis of fetal aneuploidies using QF-PCR: the egyptian study

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Summary

Background: **The most common chromosomal abnormalities identified at birth are aneuploidies of chromosome 21, 18, 13, X and Y. Prenatal diagnosis of fetal aneuploidies is routinely done by traditional cytogenetic culture; a major drawback of this technique is the long period of time required to reach a diagnosis. In this study we evaluated the QF-PCR as a rapid technique for prenatal diagnosis of common aneuploidies.**
Method: **This work was carried out on Sixty amniotic fluid samples taken from patients with one or more of the following indications: advanced maternal age (3 case), abnormal biochemical markers (6 cases), abnormal ultrasound (12 cases) or previous history of abnormal child (39 cases). Each sample was tested by QF-PCR and traditional cytogenetic. 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 QFPCR assays (S1 and S2) in order to reduce the risk of sample mishandling.**
Results: **All the QF-PCR results were successful, while there were two culture failures, only one of them was repeated. No discrepancy was seen between the results of both techniques. Fifty six samples showed normal patterns, three samples showed trisomy 21, successfully detected by both techniques and one sample showed normal pattern by QF-PCR but could not be compared to the cytogenetic due to culture failure, the pregnancy outcome of this case was a normal baby.**
Conclusion: **Our study concluded that QF-PCR is a reliable technique for prenatal diagnosis of the common chromosomal aneuploidies. It has the advantages over the cytogenetic culture of being faster with the results appearing within 24-48 hours, simpler, doesn't need a highly qualified staff, less prone to failure and more cost effective.**

Key Words: *QF-PCR, aneuploidy, STR*.

Introduction

Aneuploidies are the most frequent chromosomal abnormalities at birth. Errors in meiosis result in gametes that contain abnormal numbers of chromosomes and produce aneuploidies. Although aneuploidies are not very common, yet it is still among the most important causes of mental handicap, congenital malformation, abnormal sexual development and spontaneous abortion. The commonest autosomal trisomies are 21 (Down syndrome), 18 (Edward syndrome) and 13 (Patau syndrome). The other group of aneuploidies is the sex aneuploidies which are less sever forms, such as Turner syndrome (45, X0), Klinefelter (47, XXY)(1).
Prenatal diagnosis of chromosomal abnormalities determined by analysis of cultured cells from the amniotic fluid had its beginning in 1966. During the last 30 years, many studies has been aimed at developing of promising rapid method for prenatal diagnosis. As although cytogenetic analysis is considered as the gold standard for prenatal diagnosis, yet it has the major disadvantage of prolonged time to get final report (up to 14 days) (2,3).
In the early 1990 s,QF-PCR (quantitative fluorescent polymerase chain reaction) as a one of the molecular biological methods started to be used for the detection of major chromosomal aneuploidies aiming to provide rapid diagnosis of such chromosomal abnormalities (4-6). It is based on visualization and quantitation of specific DNA sequences (STR, short tandem repeats) using fluorescent primers (7).
QF-PCR has been confirmed to be highly sensitive and specific in detection of major chromosomal abnormalities (8-14), having the major advantage of highly throughput of samples at low cost (13,15,16).

Material and Methods

Amniotic fluid samples were collected from sixty pregnant women who were referred to the prenatal diagnosis clinic at the National Research Center, Cairo, Egypt. The selected subjects were estimated to be at high risk of having a fetal chromosomal aberration having one or more of the following criteria: advanced maternal age, which is at least 35 years old at the expected date of delivery (17), previous child or pregnancy with chromosomal abnormalities involving chromosome 21, 13, 18, X or Y or multiple congenital malformation (18), ultrasound

abnormalities and/or marker of chromosomal aneuploidy (19) and abnormal maternal serum biochemical marker (AFP and/or β-HCG) (20).

At least 20 ml of amniotic fluid were collected from each pregnant woman. The fresh sample was divided into two parts; the first part for genomic extraction followed by QF-PCR, the second part is for cytogenetic diagnosis for confirmation of the results (21). Conventional cytogenetic analyses were performed on all the prenatal samples, cultured and harvested according to standard procedures; the results were issued between 14 and 21days. Genomic DNA extraction was performed on the cell pellet obtained from 5-10 ml of amniotic fluid after centrifugation at 4500 g for 30 minutes at 4°C with the QIAamp DNA blood mini kit according to the kit's user manual (Qiagen, Germany) (22).

QF-PCR was done using Aneufast kit; the primers used in this kit are listed in Table 1.

Each sample is subjected to simultaneous analysis with two sets of markers multiplexes S1 and S2. 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 QFPCR assays (S1 and S2) 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 analyzed 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 Software (SoftGenetics, State College, PA) as previously described (7, 11, 23, 24). All prenatal samples were processed and reported within 24-48 h.

Table 1. Markers selected for QF-PCR detection of chromosome aneuploidies. Sequences producing amplicons of similar sizes are labeled with different fluorochromes to be analyzed in the same electrophoresis.

MARKER	LABEL (DYE)	CHROMOSOME LOCATION	KNOWN ALLELES IN BP
AMXY	*6-Fam*	*Xp22.1-22.31 - Yp11.2*	*X 104 Y 109*
SRY	*6-Fam*	*Yp11.2*	*Y 463*
X22	*6-Fam*	*Xq28 Yq (PAR2)*	*189-194-199-204-209-214-219-224-226-229-234-239 242-247-253*
DXYS218	*PET*	*Xp22.32 Yp11.3 (PAR1)*	*266-270-274-278-282-286-290-294*
HPRT	*6-Fam*	*Xq26.1*	*264-268-272-276-278-280-284-288-292-296-300-313*
DXS6803	*VIC*	*Xq12-Xq21.33*	*106-110-114-118-120-124-128*
DXS6809	*VIC*	*Xq*	*238-242-246-250-252-254-258-260-262-266-268-270-274*
DXS8377	*NED*	*Xq28*	*213-216-219-222-225-228-238-241-244-248-252*
SBMA	*VIC*	*Xq11.2-Xq12*	*166-169-172-175-178-181-184-187-190-193-196 199-202-205-208-211*
D21S1414	*6-Fam*	*21q21*	*328-330-334-338-342-346-350-352-354-356-358-360-362-443*
D21S1411	*VIC*	*21q22.3*	*246-262-266-274-278-282-286-290-294-298-302-306-316-319*
D21S1446	*PET*	*21q22.3-ter*	*200-204-208-212-214-218-220-224-228*
D21S1437	*VIC*	*21q21.1*	*120-124-128-132-136-140-144*
D21S1008	*6-Fam*	*21q22.1*	*196-200-204-208-212-216-220*
D21S1412	*6-Fam*	*21q22.2*	*384-388-392-396-400-406-410-414-418*
D21S1435	*PET*	*21q21*	*142-160-164-168-172-176-180-184-188*
D18S391	*VIC*	*18pter-18p11.22*	*144-148-152-156-160-164-168*
D18S390	*VIC*	*18q22.2*	*398-402-406-410-414-418-422-426-430*
D18S535	*NED*	*18q12.2*	*126-130-134-138-142-146-148-152-156*
D18S386	*NED*	*18q22.1*	*319-330-334-338-342-344-350-354-358-362-366-370-372-376-380-387*
D18S858	*PET*	*18q21.1*	*186-190-192-196-200-204*
D18S499	*6-Fam*	*18q21.32-q21.33*	*386-392-396-400-404-408*
D18S1002	*6-Fam*	*18q11.2*	*122-130-134-138-142*
D13S631	*VIC*	*13q31-32*	*192-196-200-204-208-212-215-218*
D13S634	*VIC*	*13q14.3*	*460-464-466-470-474-478-482-484-486-490-496-500*
D13S258	*NED*	*13q21*	*230-232-234-236-238-240-242-244-248-265-267-269-271-273-277-279-281*
D13S305	*PET*	*13q12.1-13q14.1*	*426-430-434-438-442-446-450-454-458*
D13S628	*6-Fam*	*13q31-q32*	*436-440-444-448-452-456-460-464*
D13S742	*VIC*	*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.

S1	S2	MXY	M21	M18	M13
AMXY	SRY	SRY	D21S1411	D18S386	D13S631
D21S1414	X22	AMXY	D21S1437	D18S391	D13S634
D21S1446	DXYS218	HPRT	D21S1412*	D18S858*	D13S742*
D13S631	HPRT	SBMA*	D21S1435*	D18S499*	D13S628*
D13S305	D21S1411	DXS6803*	D21S1008*	D18S1002*	
D18S535	D21S1437	DXS6809*			
D18S391	D13S634	DXS8377*			
	D13S258				
	D18S386				
	D18S390				

Results

Sixty amniotic fluid samples were tested in this study by QF-PCR and the results were compared to the cytogenetic results of the same sample. The maternal age of 48 (80%) out of the 60 patients involved in this study was less than 35 years old. Regarding referral cause, thirty nine patients (65%) complained of a previous history of abnormal child (twenty four patients had history of trisomy 21, twelve had history of a child with multiple congenital anomalies and three patients had history of Turner syndrome), twelve cases (20%) were referred due to an abnormal ultrasound. Six case had increased nuchal translucency, three case showed fetal bilateral ventriculomegaly, three cases showed bilateral clenched fists and club feet. Six patients (10%) were referred due to abnormal maternal serum Alfa Fetoprotein (AFP), their Multiple of Medians (MoM) ranging between 6.1 and 8.4 MoM, with highly elevated risk of aneuploidy estimated to be between 1:50 and 1:27 respectively. Three patients (5%) were referred to our clinic due to advanced maternal age (39, 40 and 41 years old) (Fig. 1).

The gestational age of the cases ranged between 12 and 28 weeks, except one case who presented to our clinic at 32 weeks of pregnancy. The total culture success rate was 90% after an average of 3-4 harvests. Two culture failures were met in this study; one of them was heavily blood stained, another clear sample was requested for re-culture with successful result. The other failed culture was due to late gestational age and most of the cells were degenerated. The culture time ranged between 14-21 days with mean and standard deviation equal to18.5 (±2.12) days.

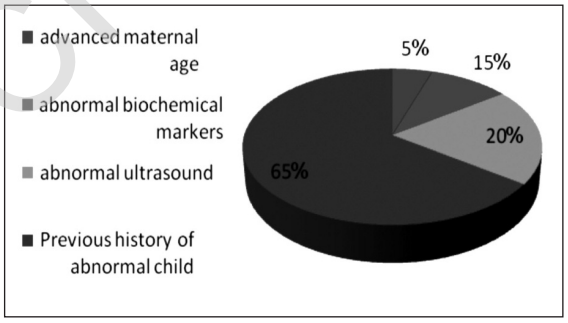

Figure 1. Pie chart showing a comparison between different causes of patients' referral.

Twenty two samples showed normal female pattern (46XX), thirty four samples showed normal male pattern (46XY), three samples showed a male pattern with trisomy 21 (47XY +21) and the result of one sample could not be obtained due to culture failure (Fig. 2).

All the samples were successfully tested by QF-PCR, results was available within 48 hours, and were in concordance with the cytogenetic results, with 100% specificity, 100% sensitivity and the diagnostic efficiency of fetal aneuploidies was 100%. The ratio between the height peaks was calculated for each marker, we assigned as normal peak ratios between 0.8 and 1.4 and abnormal ratios greater than 1.8 or less than 0.65. Two peaks with normal ratio was diagnosed as normal disomy, three peaks with ratios between 0.8 and 1.4, or two peaks with a ratio greater than 1.8 or less than 0.65 were diagnosed as trisomy. Single (homozygous) peaks were considered uninformative and were discarded. A minimum of two informative markers is required to confidently diagnose either normality or abnormality (25).

Three patterns were obtained by QF-PCR

1. Normal female pattern: Twenty two samples showed normal female patterns, with one peak appearing at the X specific locus of the AMXY marker (at 104 bp) absence of both the Y specific locus of AMXY (at 109 bp) and the SRY peaks. The X- specific HPRT marker showed normal heterozygous peaks in all except two samples, while the pseudoautosomal X22 and DXYS218 markers showed either heterozygous or homozygous patterns. The samples showed at least two normal heterozygous markers on each of the chromosomes 21, 18 and 13.

2. Normal male pattern: Thirty four samples showed normal male patterns with two peaks appearing at the AMXY for the X (104 bp) and Y (109 bp) specific loci. A single peak was present at the SRY, HPRT, while normal heterozygous peaks appearing at X22 and DXY218 markers, with the presence of at least two normal hete-

rozygous markers on each of the chromosomes 21, 18 and 13.

3. Trisomy 21 male pattern: Three sample showed amplification of the X and Y specific product of the AMXY marker, SRY marker was amplified, homozygous peak at the HPRT marker. Markers on chromosome 21 showed trisomic patterns as follows; three markers D21S1414, D21S1411 and D21S1437 showed triallelic trisomy while D21S1446 showed diallelic trisomy. The 18 and 13 specific markers showed normal patterns (Figure 3).

Inconclusive results: One sample showed inconclusive result for chromosome 21 with only one heterozygous marker D21S1414, one homozygous marker D21S1446, while D21S1411 and D21S1437 failed to be amplified. The other markers on chromosome 18, 13, X and Y showed normal male pattern. The same sample was retested with the chromosome 21 extra markers. The extra markers (D21S1008 D21S1412, D21S1437 and D21S1411) showed normal heterozygous peaks.

Discussion

During the past decades, there has been a considerable progress in further refining the non-invasive methods for the prenatal detection of fetal diseases. Biochemical and ultrasound tests have become standard procedures

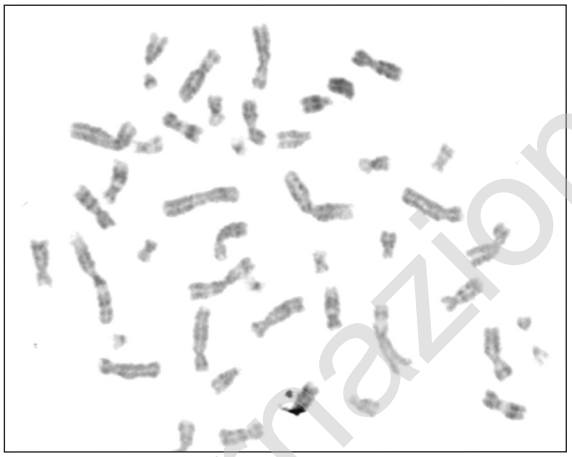

Figure 2. Figure showing a G-banding metaphase of normal female.

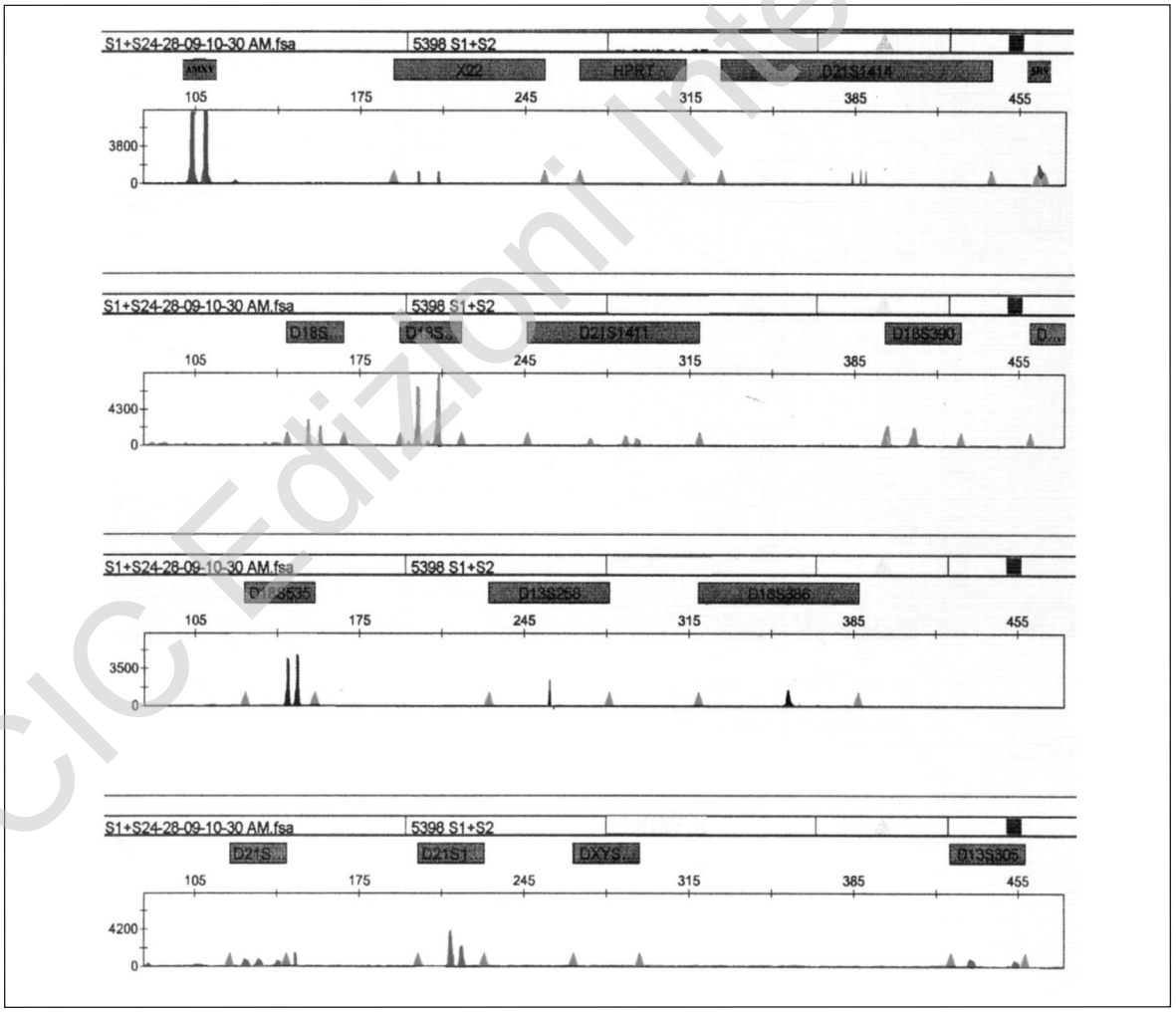

Figure 3. Trisomy 21 male pattern: the D21S1414, D21S1411 and D21S1437 markers show triallelic trisomic peaks, the D21S1446 shows diallelic trisomic peaks. The chromosome 18 and 13 specific markers show normal figures. The X and Y chromosome specific AMXY markers amplified, SRY product present, other sex chromosome markers show heterozygous diallelic figure.

for screening for fetal chromosomal abnormalities. Both approaches imply that a high proportion of tested mothers are told that their fetuses may have a major chromosome disorder and that an invasive procedure is required to confirm the diagnosis (26).

In our study, the patients who underwent amniocentesis were referred to our clinic due to different causes, the most common of which was a previous history of an abnormal child (39 cases), the next common cause of referral by an obstetrician due to abnormal ultrasound findings (12 cases), followed by abnormal biochemical markers (6 cases) and the least common cause of referral was the advanced maternal age (3 cases). This ranking was different from that presented by Cirigliano et al. (27) showing that in the Western countries, the most common cause of patients' referral for amniocentesis was the advanced maternal age, followed by abnormal biochemical markers then abnormal ultrasound. We estimate that the advanced maternal age, being the first cause of referral in the west and the last in our study, might reflect the low awareness among our population of the increasing risk of aneuploidies with age, and lack of knowledge of the prenatal diagnosis tests as a routine antenatal test.

Twenty four patients had previous history of Down syndrome child, these cases showed both normal karyotype and QF-PCR result; none of these patients showed recurrent Down syndrome. This is in agreement with Cui et al. (28) who stated that the risk of the recurrence of a Down syndrome after the birth of an affected child is only 1%. The authors reported that recurrent trisomy 21 may be owing to chance alone because of the maternal age-associated risk, parental gonadal mosaicism for trisomy, or factors associated with an increased risk of meiotic error.

Twenty cases underwent the test due to abnormal ultrasound, the three case showed clenched fists and club feet with suspected trisomy 18, however, the cytogenetic and QF-PCR showed normal male pattern, showing that the presented ultrasound findings were not associated with trisomy 18 as expected, and that they may be associated with another chromosomal abnormality that could not be diagnosed neither by QF-PCR nor by the traditional karyotype. In these cases QF-PCR excluded aneuploidy but did not help the diagnosis; however, it also shows that waiting for the long term culture result would not give any further information. Therefore, we assume that the diagnosis should be based on both the clinical and laboratory findings, taking into consideration that QF-PCR is mainly used for the diagnosis of fetal aneuploidies.

Another three cases with abnormal ultrasound showed severe fetal ventriculomegaly by ultrasound, the abnormality that could be associated with fetal aneuploidies especially Down syndrome (in 75% of cases). These cases showed no chromosomal abnormality when tested by both QF-PCR and traditional cytogenetics. This is the second time in the study where an abnormality in the ultrasound suggestive of fetal aneuploidy had a normal karyotype.

This was also mentioned by Breeze et al. (29) who stated that fetal ventriculomegaly is not necessarily associated with fetal aneuploidy it may occur for a number of other reasons, such as impaired outflow or absorption of cerebrospinal fluid from the ventricles that may be due to congenital malformation of the interventricular foramen.

The remaining six cases with abnormal ultrasound showed increased nuchal translucency, three of them gave normal results, while the other three were associated with abnormal maternal serum AFP and was diagnosed as trisomy 21 by both QF-PCR and cytogenetics. We thus found that isolated increased NT is not necessarily associated with fetal aneuploidy, but the risk is higher when it is associated with other parameters such as biochemical markers. This is in agreement with Tamsel et al. (30) who stated that increased NT is present in 80% of Down syndrome, but could also be associated with other chromosomal defects and Bilardo et al. (31) who reported that increased NT could be associated with normal karyotype.

From the analysis of the twelve cases with ultrasound abnormalities, presented above, we can define them as a particular risk category where it is possible to find a genetic defect the QF-PCR is not designed to detect. A close attention should be given for this category, the cytogenetic culture is also important to be done to detect possible structural chromosomal abnormalities, taking into consideration that sub-microscopic defects may still be undiagnosed by both techniques.

Nine cases had AFP concentration ranging between 6.1 and 8.4 MoM. Six cases showed both normal karyotype and QF-PCR result, while the other three cases had an associated increased NT (previously mentioned) and showed trisomy 21 children both by cytogenetics and QF-PCR. These cases showed that an abnormal biochemical marker, especially when used alone is not necessarily associated with fetal abnormality.

The culture success rate among the tested samples was 90%, two failures were met in this study. The first one was heavily blood stained. The cells failed to adhere to the surface and no colonies were formed. We estimate that the excessive blood cells in the sample, even after its treatment with distilled water to induce red blood cells rupture, interfered with the adherence of the cells and was the main cause of culture failure. The QF-PCR result could however be obtained, another sample was requested for re-culture. The second sample was clear with successful result similar to that of the QF-PCR. This is in disagreement with Sikkema-Raddatz (32) who stated the impact of bloody amniotic fluid was an extended culture time rather than culture failure. However, the culture type presented in this study was different from ours, as they used in situ short term culture, while we used long term culture with flaskettes and flat sided tubes. The other culture failure was probably due to the late gestational age (32 weeks), and a small number of viable cells could be seen in this sample, while most of the cells were degenerated.

As described before, there was no discrepancy between the traditional cytogenetic and QF-PCR results, no false positive or false negative results. We estimate 100% specificity and 100% sensitivity of the technique for the diagnosis of chromosomal aneuploidies. A slightly different rate was presented by Cirigliano et al. (27) with 100% specificity and 99.7% sensitivity of the technique, the 0.3% difference may be attributed to the much larger number of samples studied by the authors. On the contrary, Waters et al. (33) reported three cases with discrepancy between QF-PCR and cultured cells. However, the authors used chorionic villous samples which is subjected to false results as the abnormality may be confi-

ned to the placenta, while the baby is normal (34). In our study we used a different type of sample (amniotic fluid) which is not subjected to this problem.

Although the previous studies evaluating the QF-PCR, the authors assured the reliability of this test for prenatal diagnosis of chromosomal aneuploidies (25, 35, 36), however the informativity of the markers used may differ from one population to another. The Middle Eastern countries are characterized by a high rate of consanguinity estimated around 25% and tend to display a lower rate of heterozygosity. Therefore we evaluated the informativity of the previously established markers on our population, and hence their applicability in the prenatal diagnosis.

We found that two markers on chromosome 21, D21S1414 and D21S1411 showed the highest rate of heterozygosity 0.95 and 0.889 respectively, with only one homozygous result seen with D21S1414 and two homozygous peaks seen with D21S1411. The first marker D21S1414 was previously used by Cirigliano et al. (12) and Diego-Alvarez et al. (37), and showed high rate of heterozygosity and reliability in the diagnosis of trisomy 21. Another value of this marker is that it maps for the long arm of chromosome 21 flanking the Down's syndrome critical region, thus allowing the detection of most partial trisomies due to unbalanced translocations (11). In our study we did not meet any case of partial trisomy.

The high reliability of the second marker D21S1411 was also in concordance with Brown et al., (36). This marker was co-amplified with the HPRT in our study, which allows its use as an internal control for the HPRT marker and vice versa, as it has been shown that when the ratios of fluorescent activities between several X-linked and autosomal polymorphic sequences were compared, only D21S1411 STR was found to provide an accurate measure of the X chromosome present in a sample as these sequences generate alleles of very similar size, but they can be readily identified using primers labeled with different fluorochromes (24). We benefited of this fact in one case where the AMXY showed one X-specific peak, and all the X-specific markers were homozygous. To differentiate between a normal homozygous female and X monosomy we calculated the ratio between the fluorescent product of the two markers HPRT: D21S1411 which was 2 : 1 : 1.1 thus showing a normal female pattern, this was also confirmed by the cytogenetic results.

The chromosome 18 specific markers used in the present study are D18S390, D18S391, D18S535 and D18S386. These markers performed well with 0.778, 0.75, 0.75 and 0.737 heterozygosity respectively. The combination of the four markers allowed an informative diagnosis in all the tested samples. This was in agreement with Mann et al. (1), who reported the high reliability of the same markers in their study.

In our study all the samples showed at least 2 heterozygous peaks for chromosome 13 markers D13S258, D13S634, D13S631, and D13S305. The markers showed high heterozygosity rate of 0.889, 0.824, 0.789 and 0.778 respectively. There was no need to use the extra markers. The high reliability of these markers was in concordance with Diego-Alvarez et al. (37) who reported that there were no uninformative results when using these markers.

The sex chromosome markers performed well in the present study, allowing the detection of the fetal sex as well as the exclusion of sex chromosome aneuploidies. The sex determination was based mainly on the non-polymorphic AMXY marker present on the X and Y chromosomes and the Y chromosome specific marker SRY, with successful diagnosis in all the tested samples when compared to the cytogenetic results. This is in agreement with Onay et al. (38) who used the same markers for fetal sexing with high reliability.

In this study, the expenses of the test done by QF-PCR was found to be less than that done by cytogenetics, this was in agreement with Mann et al. (1) and Dudarewicz et al. (19). The authors stated that the use of cytogenetic test require is more expensive especially if the culture fails and the test needs to be repeated, the expenses are found to be low when the markers are prepared in the lab. Although we used readymade multiplex, the test done by QF-PCR was still more cost effective, as the results were informative from the first time in all except one case where the test was repeated with additional markers for chromosome 21.The additional markers are included in the used kit, with the extra cost being minor and only limited to the rerun of the sample. On the other hand, most of the successful cultures gave results after the second or third harvest with more time and expenses consumed, in addition to the expenses of the repeated culture due to culture failure.

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