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Detection of Numerical Aneuploidy of Chromosomes X, Y, 13, 18 and 21 in 100 Blood and Fetals Samples by QF-PCR Method

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Abstract

Objective: Determination of parents to avoid giving birth to a child with any anomalies has increased the demand for prenatal diagnosis. Two most important criteria of any prenatal diagnosis procedure are accuracy, and speed. They have to minimize burden and anxiety for families. One of the main tests requested during pregnancies is testing for chromosomal abnormalities. This study was carried out with the aim of investigating the use of a rapid diagnosis test for detecting chromosomal numerical aneuploidy in blood and fetal samples, and also to compare the outcomes with cytogenetic method.

Materials and methods: In this study, 100 samples from high risk pregnancies or affected individuals, comprising 12 chorionic villi (CV), 43 amniotic fluids (AF) and 45 blood samples were analyzed by QF-PCR. The samples were amplified using the specific microsatellite markers (STRs) for chromosomes X, Y, 13, 18 and 21. The sample analysis was performed based on the peak type of the PCR products, and the results were compared with the cytogenetic findings.

Results: In total 26 samples were normal and 74 were diagnosed as aneuploids. Eight sex chromosome aberrations (three 45, X; three 47, XXY; one 47, XXX and one 46, XY (female phenotype), 65 numerical aberrations of X, Y, 13, 18 and 21 chromosomes and one triploidy were recognized. The QF-PCR data were compared with the karyotype results and showed complete concordance.

Conclusion: This study showed that QF-PCR method is definitely superior, due to its advantages and few drawbacks in diagnosis of numerical chromosomal aberrations. Low costs and high speed of analysis as well as its automaticity are among the most important advantages of this method.

Considering more than 99.4% accuracy of the QF-PCR method (compared with cytogenetics) and the time required to do cytogenetic analysis, QF-PCR is the method of choice for aneuploidy testing.

Keywords: Numerical aneuploidy; Prenatal diagnosis; QF-PCR; Microsatellite; Cytogenetics

Introduction

Aneuploidy is the most common chromosomal aberration with clinical importance in humans. It is of high frequency in embryos and exists in 3 to 4% of recognized pregnancies and 1 in 160 live births [1]. Probability of aneuploidy occurrence increases with maternal age. Aberrations of chromosomes X, Y, 13, 18 and 21 comprise about 95% of all chromosomal disorders in newborns. Meanwhile, trisomies of chromosomes 13, 18 and 21 are more important and trisomy 21 is the most common [2]. Screening for genetic diseases is a well-accepted procedure as a preventive solution in many countries. Biochemical analysis is the primary stage of any aneuploidy screening in pregnancies. Ultrasonography is usually used in conjunction with biochemical tests to find high risk pregnancies. The accuracy of these two methods has been reported to be between 79-90% [3].

Though these two methods are non-invasive, due to low accuracy, alternative methods are used to increase the detection rate [4,5]. The most widely used method is fetal karyotyping. Cytogenetic analysis usually requires 15 to 25 days, a time of extreme anxiety for the couple and their family. In many cases, late therapeutic abortions may be risky. Furthermore, karyotype preparation and analysis are expensive and difficult [6-8]. Therefore Rapid Aneuploidy Diagnosis (RAD) has become a necessity. One such method is the use of FISH (Fluorescence In Situ Hybridization). FISH is a molecular method in which chromosome specific probes are labeled with fluorescent materials and then are hybridized to chromosome spreads laid on slides [9-11]. The targets are specific sequences of DNA, to which probe will bind. In aneuploidy FISH, probes are specifically designed for the chromosomes X, Y, 13, 18 and 21 [10]. FISH, though capable of diagnosing the chromosomal aberration using the nucleated cells, is expensive, tedious, requires dedicated probes and is not so easy to get good results in somewhat inexperienced hand. Another rapid molecular test is the use of STR (short tandem repeat) based quantitative fluorescent PCR (QF-PCR) [12]. QF-PCR as its stands for is a method in which by means of fluorescent based PCR one can quantitate specific DNA copy number

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or DNA type, but is more used for detecting chromosomal aneuploidy in prenatal testing.

Publication of a number of papers regarding evaluation of the STRs for the gene mapping proved their diagnostic application value for identifying a group of aneuploidies [13,14]. Nowadays QF-PCR is preferred over FISH. Markers used in aneuploidy QF-PCR are located in regions of tested chromosomes with polymorphic alleles [15]. The microsatellites used in this method are 3-5 base pairs long [16].

Though cytogenetic method is still the golden choice, rapid methods are preferable since the risk of abortion-related mortality increases with gestational age [17]. Nowadays, QF-PCR is used in prenatal diagnosis centers in Europe for diagnosing the most important chromosomal numerical aberrations [15,18-24]. In countries like the UK, new screening programs for Down Syndrome (DS) need not include karyotyping and can offer diagnosis with RAD as a standalone approach [20,25]. QF-PCR is the most preferable and easy to use method as a RAD method.

In the present study, aneuploidies were studied in 100 chorionic villus (CV) samples, amniotic fluid (AF) samples and blood samples collected from the pregnant women and patients and the results obtained were compared with cytogenetic analysis results.

Materials and Methods

The samples were supplied from several Welfare Centers and medical genetics laboratories. The samples included blood and fetal samples (CV and AF). All samples were used after signing of informed consent forms. The criteria for taking samples for prenatal diagnosis included advanced maternal age (n=11), abnormal fetal ultrasonographic signs with or without advanced maternal age (n=9), positive results following maternal blood biochemical screening (n=25), previous record of chromosomal aberration (n=9) and abnormal karyotype of parents (n=1). The blood samples (n=45) belonging to patients who had been referred to the medical genetics centers for confirmation of DS or other chromosomal anomalies or placed in the national Welfare Centers. Blood samples, of about 2 ml, were taken from the affected persons and were spotted onto DNA Banking Cards (DBC) (Kawsar Biotech Companies, KBC, Tehran, Iran). To extract the DNA, a 1.5 mm diameter circles were cut using a Micropunch (KBC, Tehran, Iran) and rinsed using DBC extraction buffer (KBC, Tehran, Iran) for 2-3 times. The discs were used directly after the final rinse. DNA from chorionic villi (CV) were extracted using trypsin and using method described [26]. DNA from amniotic fluid (AF) samples, were extracted using the method described elsewhere [27]. QF-PCR was done using Aneufast kit as recommended (Genomed AG, Switzerland) (Table 1). For all cases, initially S1 and S2 were used and if any confirmation was needed chromosome specific kits (i.e. MXY, M12, M18, M13 for X and Y, 13, 18 and 21 chromosomes respectively) were used according to manufacturer protocol.

For performing QF-PCR either one disc from the DBC or 5 microliter (µl) DNA was added to 10 µl of Aneufast multiplex mix. PCR was done as an initial denaturation for 15 minutes, amplification using 28 cycles at 95°C for 40 sec, 58°C for 80 sec, and 72°C for 40 sec, and final extension for 30 sec, at 60°C. About 1.5 µl of each of the PCR products together with 0.3 µl of the size standard of GeneScan™-500 LIZ™ was added to 40 µl of Hi-Di Formamide. The samples were denatured for 2 minutes at 95°C and loaded into ABI 3130 XL Genetic Analyzer (ABI, US). Cytogenetics was done as described [28]. Analysis was done using automated cytogenetics platform CytoVision and its Software (Wetzlar, Germany).

Results

In this study, 100 samples including 45 blood, 43 AF and 12 CV samples were analyzed. The DNA extractions were successful in all the blood and CV samples, but it was unsuccessful in two of the AF samples due to high contamination by maternal blood. These two gave poor and unanalyzable results but subsequent purification using KBC DNA cleanup kit (KBC, Tehran, Iran) and repeating the QF-PCR gave good results. In general, the DNA extraction from the chorionic villus samples was much simpler and more accurate than the AF, since the maternal blood and blood clots can easily be removed prior to DNA extraction from the CVs. Using the QF-PCR method the analysis results of 90% of the samples were available within 48 hours but 10% of the samples were re-analyzed because of uncertainty. Cytogenetic analysis was performed within two-four weeks. Results of cytogenetics and QF-PCR for each sample were analyzed independently and they were compared after the end of the study. Several numerical chromosome abnormalities were observed and are summarized in Table 2.

Diagnosing normal samples

Presence of at least two different STR peak for each chromosome with a 1:1 ratio proves normality. All the blood samples in this analysis had been taken from the individuals with chromosome aneuploidy; so there were no normal cases. 10 samples (83.3%) out of 12 CV and 16 samples (37.2%) out of 43 AF were normal. Fetal sexing and the numbers of sex chromosomes were determined through amplification of the non-polymorphic sequences of amelogenin gene. This region give two differing bands one for the Y and the other one for the X chromosome. When both bands are present then the sample is regarded

Marker	Label	Chromosome Location
AMXY	6-Fam	Xp22.1-22.31-Yp11.2
SRY	6-Fam	Yp11.2
X22	6-Fam	Xq28 Yq(PAR2)
DXYS218	PET	Xp22.32 Yp11.3(PAR1)
HPRT	6-Fam	Xq26.1
DXS6803	VIC	Xq12-Xp21.33
DXS6809	VIC	Xp
DXS8377	NED	Xq28
SBMA	VIC	Xq11.2-Xq12
D21S1414	6-Fam	21q21
D21S1411	VIC	21q22.3
D21S1446	PET	21q22.3-ter
D21S1437	VIC	21q21.1
D21S1008	6-Fam	21q22.1
D21S1412	6-Fam	21q22.2
D21S1435	PET	21q21
D18S391	VIC	18pter-18p11.22
D18S390	VIC	18q22.2
D18S535	NED	18q12.2
D18S386	NED	18q22.1
D18S858	PET	18q21.1
D18S499	6-Fam	18q21.32-q21.33
D18S1002	6-Fam	18q11.2
D13S631	VIC	13q31-32
D13S634	VIC	13q14.3
D13S258	NED	13q21
D13S305	PET	13q12.1-13q14.1
D13S628	6-Fam	13q31-q32
D13S742	VIC	13q12.12

Table 1: STR locations and labeling information for the Aneufast kit.

Karyotype	Cytogenetics	QF-PCR
48,XX;46,XY	27	26
47,XX+21;47,XY+21	60	60
47,XX+18;47,XY+18	4	4
45,XX-18;45,XY-18	1	1
69,XXX;69,XXY	0	1
45,X	3	3
47,XXY	3	3
47,XXX	1	1
46,XY (Female Phenotype)	1	1
Total abnormalities	73	74
Sensitivity(%)		99
Specificity(%)		100
PPV(%)		99
NPV (%)		100
Total 100		

Table 2: Results of 100 samples tested by QF-PCR and cytogenetic methods.

male gender otherwise the gender is regarded female. Other STR markers confirm the above results and may show other forms of sex chromosome abnormality like XXY which will not be detected using the amelogenin gene. Detection of fetal sex was correctly performed in all the blood samples, and no difference was observed between the cytogenetic and the QF-PCR results.

Autosomal AD was possible by the presence of at least three different STRs as triallelic peaks (three alleles equal in size) or in diallelic trisomy state (two alleles, one twice the size of the other) for each chromosome proving it being a trisomy. 41 samples (91.1%) out of 45 blood samples were recognized as trisomy 21. 16 samples (37.2%) out of 43 amniotic fluid samples had trisomy 21 and three cases (6.98%) had trisomy 18. None had trisomy 13.

Sex chromosome AD

Sex chromosomes were correctly recognized in all samples. One case (2.22%) of Turner syndrome (XO) (X monosomy) and two cases (4.44%) of Klinefelter (XXY syndrome) were recognized in blood samples. In addition, one case (2.32%) of Turner syndrome, one case of the Klinefelter syndrome and one cases of X trisomy (XXX) were observed in amniotic fluid samples.

In two AF samples, the QF result was different from that of cytogenetics. One sample was diagnosed as trisomy 21, while the cytogenetic analysis result recognized it as normal. This could be due to mosaicism and preferential growth of normal cells during AF culturing as seen before [29].

The other sample was poorly identified as triploidy or tetraploidy by QF method, while cytogenetic showed that the fetus had trisomy 21. Maternal blood contamination may be the caused for controversial QF result. DNA extracted from the AF cultured cells confirmed cytogenetic result.

One sample detected to have mosaic triploidy by QF-PCR which failed to provide cytogenetics result due to inability of the cells to grow. There may other causes as well but this is more likely [29].

Discussion

In normal individuals who are heterozygous from STRs, an equal amount of fluorescent is created for both alleles; so the proportion between the areas and heights of the peaks will be almost 1:1. In normal individuals, whose STRs' alleles are homozygous, would have the same repeat number and size; so quantification would be impossible, and the marker is not useful for the analysis. Nevertheless the markers have been chosen as such that it is very rare to have a normal person to be homozygote for all markers in the general kit and chromosome specific kit. In a trisomy sample, three versions of a chromosome are diagnosed; consequently, the chromosome STRs usually show three peaks with almost identical fluorescent intensity and proportion of about 1:1:1 between the areas (triallelic trisomy) (Figure 1). In a trisomic situation when two chromosomes have similar repeat unit (equal PCR product size) and one a different size, the quantitative PCR produces two imbalanced peaks with an area proportion of 2:1 (diallelic trisomy) (e.g. D21S1435 marker in Figure 1).

QF-PCR is a simple, rapid and cost effective method for prenatal diagnosis of common chromosomal aneuploidies (e.g. 21, 18, 13, X and Y). The result can be ready within 24-48 hours after sampling while cytogenetics usually requires cell culturing and it usually takes more than 14 days to produce enough cells to make cytogenetic preparation possible. When human labor and other costs are compared for both methods then the cost for the QF is almost half of the cytogenetics [30]. Another advantage of QF-PCR is the reduction of waiting time for results by the families. Usually families are under immense stress after giving the fetal sample and waiting for results (personal observation).

One may argue that cytogenetic is more accurate therefore the accuracy should not be compromised over speed or cost. This point has been extensively reviewed and two major policies are under practice [30]. In the UK, the use of RAD methods are sufficient for aneuploidy testing during pregnancies when only aneuploidy screening is the aim. Nevertheless, in the USA "a joint statement by the American College of Medical Genetics and the American Society for Human Genetics reaffirmed that all RAD test results must be followed up with karyotyping" [30,31]. QF-PCR is regarded the most cost effective and preferable method for AD.

In our study the QF-PCR diagnosed 100% of the trisomies 13, 18 and 21 and sex chromosome aneuploidies without false-negative results, and the fetal detection was perfectly done in all the samples (Table 2). It however, could not easily detect mosaicism (Table 2). This is a particular problem when one type of karyotype is less represented (e.g. less than 1:4-6 ratios). The STR markers in the Aneufast kit were 100% informative for all the samples. In at least one sample maternal blood contamination gave unacceptable QF-PCR result. More care

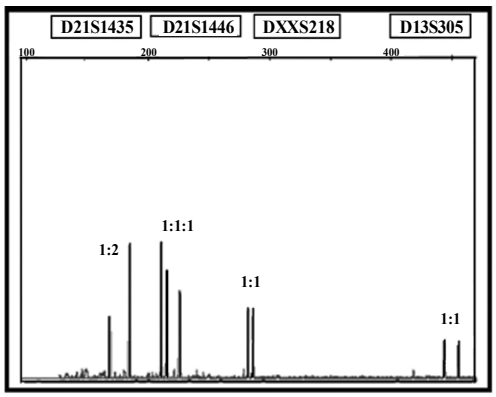

Figure 1: Ratio types among the peaks. In D21S1435 area, a peak is twice the other, indicating diallelic trisomy In D21S1446 three peaks are seen, which show typical triallelic trisomy. In DXYS218 and D13S305 areas, two peaks with identical sizes are seen, showing the normal state in such areas.

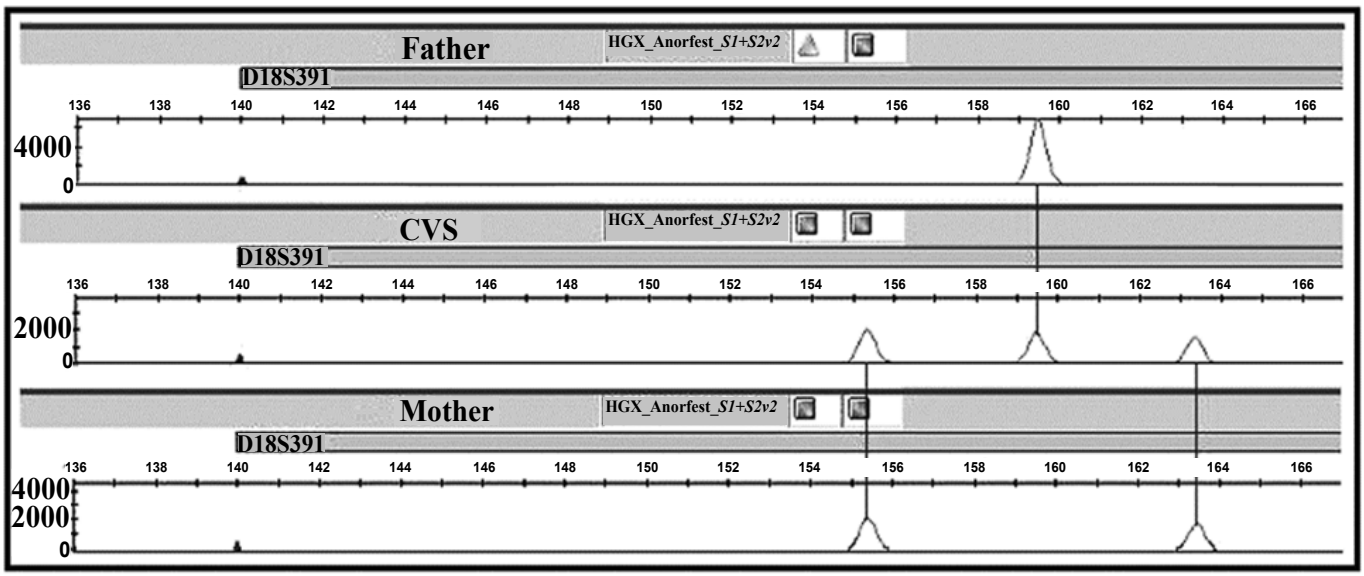

Figure 2: This figure shows that the fetal sample has three peaks for marker D18S391 on chromosome 18, two alleles belongs to the mother. It means that the extra chromosome has been as a result of nondisjunction of maternal chromosome at the first mitotic division.

has to be employed during AF sampling to reduce maternal blood contamination of the AF. When maternal blood is obscuring the results, parallel testing of maternal DNA may resolve the problem or one has to rely on cytogenetic results. Our strategy in this study was that we would culture all AF samples and also perform the QF-PCR. If QF was successful and clear result was obtained then we would inform the family about the result but wait for the cytogenetic result to see if it would confirm the QF-PCR outcome. This strategy would be violated (i.e. relying only on the QF-PCR) if the gestational age is as such that the cytogenetic results would become ready after the legal time limit for therapeutic abortion (end of 18th weeks in Iran).

Regarding limitations of diagnosing chromosomal aberration, FISH method is similar to the QF-PCR method, while the QF-PCR has several advantages over the FISH method. Misdiagnosis in contaminated samples is less in QF-PCR than FISH [32]. Costs and complexity of FISH are more than QF-PCR. The karyotype methods also enjoy high accuracy, while being time consuming due to the need for cell culture. Numerous studies have been performed to determine the accuracy of chromosomal disorder diagnosis. Cirigliano et al. performed an investigation on a large number of the samples during a nine year period [33]. Based on these studies, the QF-PCR method was found to be capable of diagnosing the chromosomal aneuploidies of chromosomes X, Y, 13, 18 and 21 with 100% accuracy; also above 95% of the chromosomal aberrations were diagnosed using the clinical symptoms with ease and termination of pregnancy could be performed without waiting for cytogenetic analysis results [33]. In addition, in a report recently published a sum up of the study results of several scientists on QF-PCR method over a 15-year period has been given [9,34]. The group announced that the pregnant women can perform this analysis as an alternative to the cytogenetic methods. The major disadvantage of this method is its inability to detect mosaic and numerical aneuploidy of the chromosomes other than X, Y, 13, 18 and 21, as well as its inability to detect structural aberrations [9,34]. However, other chromosomal abnormalities (CA) are rare among women who are under general screening program (with no prior history or complication before screening). QF method is also capable of detecting the origin of extra chromosome (Figure 2).

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