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Quantitative Fluorescence Polymerase Chain Reaction (QF-PCR) for Prenatal Diagnosis of Chromosomal Aneuploidies

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ABSTRACT Genomic aneuploidy is a common cause of human genetic disorders and cytogenetic analysis of metaphase karyotypes remain the standard method to identify aneuploidies and balanced translocations. Quantitative Fluorescence PCR (QF-PCR) is an alternative method in which DNA polymorphic markers on chromosomes, is used to determine the presence of different alleles**.** The assay based on the use of informative polymorphic small tandem repeat (STR) markers and the availability of parental DNA, is employed for prenatal and postnatal diagnosis of aneuploidies of chromosomes 13, 18, 21, X and Y. DNA isolated from fetal cells of amniotic fluid sample, chorionic villus sample, fetal trophoblast cells from endocervical lavage and neonatal blood are all used for the investigation of chromosomal copy number variations. The QF-PCR assay uses fluorescent labelled primers of STR markers that are analyzed after fragment length separation in capillary gel electrophoresis. The determination of the meiotic origin of aneuploidy or the post zygotic mitotic origin could also be done in most cases. Though testing of prenatal samples is complicated by limited sample quantity, variable sample quality, mosaicism and maternal cell contamination- use of parental samples and other measures can overcome most of these limitations. The QF-PCR technique serves as a very useful preliminary test to reduce parental anxiety within a short duration, and to accelerate therapeutic intervention.

INTRODUCTION

Aneuploidy, or the loss or gain of a chromosome in a diploid complement is a common cause of human genetic disorders. Pregnancies with chromosomal aneuploidies that survive to term including trisomies of autosomal chromosomes 13, 18 and 21 constitute approximately 90 percent of chromosomal abnormalities with a severe phenotype (Mann et al. 2008). The term aneuploidy was earlier restricted to indicate supernumerary copies of whole chromosomes as in trisomy, or the absence of chromosomes namely, monosomy. Currently the use of this term is extended to include deletions or duplications of subchromosomal regions. Trisomies can thus be divided into four categories based on the size of the triplicated genomic region. They are complete (whole-chromosome) trisomies resulting from meiotic or mitotic non-disjunction that account for up to 0.5% of live births; partial (segmental) trisomies involving a genomic region of more than one chromosomal band (usually larger than 5 Mb) resulting from abnormal meiosis and segregation in individuals with balanced chromosomal rearrangements which is much less frequent than whole-chromosome trisomies; microtrisomies (segmental duplication) that are partial trisomy of a genomic segment that is shorter than 3–5 Mb; and the duplications of only one gene or one functional genomic element that can also be pathogenic (Antonorakis et al. 2004).

A great deal of information pertaining to the pathobiology of the constitutional trisomies, the supernumerary chromosomes that occur in all cells, including trisomies 13, 18 and 21 have come from the study of Down syndrome. This disorder which is a model for chromosomal aneuploidies has itself been characterized by wide ranging phenotypic variability on account of the variability of the human chromosome 21. Genetic structure underlying this variability of the chromosome 21 with respect to the SNPs involved in gene expression variation has been mapped through chromo-some wide linkage disequilibrium studies. Trisomy 18, also known as Edward syndrome, first described in 1960, is the most common trisomy behind Down syndrome, occurring in 1:3000 conceptions and 1:6-8000 live births. Like Down syndrome, the likelihood of its occurrence increases with the age of the mother. This disorder shows a higher incidence for females believed to be due to a higher prenatal mortality of males. Trisomy 13, also called Patau syndrome, though of rarer occurrence with an incidence of 1 in 16,000 live births, causes much more severe and life threatening medical

conditions that allows only about 5 to 10 percent of the affected children to live up to the first year of their life. Currently, based on the discovery of several factors, the understanding provided is that there is a risk of aneuploidy for the fetus in every pregnancy as opposed to the earlier assessment of maternal age and family history as the criteria. Conse-quently prenatal screening for the chromosomal aberrations and the practice of clinical genetic counseling are of great significance.

Metaphase karyotype analysis by cytogenetic techniques remains the standard practice to identify all aneuploidies and balanced translocations. Other methods available for testing are fluorescence in situ hybridization (FISH), Southern hybridization, loss of heterozygosity (LOH) assays, microarray technology, comparative genomic hybridization (CGH), automated nuclear DNA cytometry, several modifications of polymerase chain reaction (PCR), quantitative fluorescence polymerase chain reaction (QF-PCR) etc., with variable practical feasibility for prenatal diagnosis in a routine clinical laboratory setting (Dudarewicz et al. 2005; Hahn and Jackson 2008). Currently a few of the above mentioned techniques are used in clinical practice as preliminary tests to reduce the considerable 'maternal anxiety' while the more time consuming cytogenetic analysis of the karyotype is undertaken. The discussed techniques are so far not used as stand-alone tests, but some of them are routinely applied concurrently with the cytogenetic karyotyping as a preliminary test that shortens the waiting time.

The method that is now widely used for the prenatal diagnosis is quantitative fluorescence PCR (QF-PCR), in which DNA polymorphic markers (microsatellites) on autosomes 13, 18 or 21 or sex chromosomes is used to determine the presence of three different alleles. This method relies on informative markers and the availability of parental DNA. There are several characteristics and advantages of the diagnostic procedures using the techniques that are listed below:

(1) Rapid diagnosis of aneuploidy.
(2) It is a reliable molecular biological method, which uses chromosomal polymorphic di, tri or tetra- nucleotide STR (Small Tandem Repeats) markers for the detection of major chromosomal aneuploidies in prenatal and postnatal diagnosis involving chromosomes 13, 18, 21, X and Y in affected offspring and their parents.
(3) Parental origin of the aneuploidy and determination of origin of non-disjunction in meiosis I or II can also be evaluated by means of QF-PCR.
(4) In case of aneuploidy either a triallelic peak pattern or a diallelic peak pattern showing quantitative difference can be observed.
(5) Further the parental origin of the supernumerary chromosome can be shown in most cases by means of QF -PCR.
(6) The determination of the meiotic origin of aneuploidy or the post zygotic mitotic origin could also be done in some familial cases.
(7) The testing of prenatal samples is complicated by limited sample quantity, variable sample quality, mosaicism and maternal cell contamination. Stringently controlled QF-PCR can overcome most of these limitations.

METHODS

The samples were collected from the Department of Obstetrics and Gynecology at the Kasturba Hospital, Manipal, from pregnant women of high risk groups such as those presented with a family history of chromosomal disorders or with advanced maternal age, among others, for our study of QF-PCR method for diagnosis of trisomies. DNA isolated from the fetal cells obtained from a sample of amniotic fluid (8-10 ml), chorionic villus sample (2-5 mm) or trophoblast cells from endocervical lavage, were analysed using chromosome specific STR markers (Table 1) by QF-PCR. Multiplex QF-PCR assays were set up initially for a set of two STR markers for each chromosome. Where the markers showed homozygosity, additional markers were included in further assays. DNA isolated from maternal blood samples (2-5 ml in EDTA) were analysed to exclude maternal contamination in some cases to clarify the result. If unused, the maternal blood was discarded after the PCR test results were analysed. A major subset of pregnancies were also tested by full karyotyping based on clinical indications. Highly polymorphic STR markers were used for PCR amplification with labeled primers and the results after fragment length separation in capillary gel electrophoresis (ABI, Genetic analyzer 3130, with Gene Scan Software for DNA fragment sizing) are presented as a graph and ratios are calculated using a spreadsheet. A few of the oligonucleotide primers used in the amplification of the marker loci in our

Table 1: The chromosomal markers for the detection of aneuploidy and the oligonucleotide primer sequences used for their amplification along with the physical conditions for the polymerase chain reaction

S. No.	*Marker*	*Fluorescent Label*	*Primer Sequences (Forward and Reverse)*	*Size of PCR amplicon (bp)*	*PCR Conditions*
1	D21S11	FAM	5’ TATGTGAGTCAATTCCCCAAGTGA 3’ 5’ GTTGTATTAGTCAATGTTCTCCAG 3’	220-235	96°C - 05min; 94°C -45 Sec, 60°C-45 Sec; 72°C -1min; [25 cycles] 72°C -5min
2	D21S1270	NED	5’ CTATCCCACTGTATTATTCAGGGCTGA 3’ 5’ GTCTCCAGGTTGCAGGTGACA 3’	290-310	96°C - 05min; 94°C -45 Sec, 62°C-45 Sec; 72°C -1min; [25 cycles] 72°C -5min
3	D21S1414	FAM	5’ GGCACCCAGTAAAAAATTACT 3’ 5’ CTGTCTGTCTGTCTGTCTACT 3’	285-300	96°C - 05min; 94°C -45 Sec, 60°C-45 Sec; 72°C -1min; [25 cycles] 72°C -5min
4	D21S167	FAM	5’ TCCTTCCATGTACTCTGCA 3’ 5’ TGCCCTGAAGCACATGTGT 3’	150-160	96°C - 05min; 94°C -45 Sec, 60°C-45 Sec; 72°C -1min; [25 cycles] 72°C -5min
5	D13S628	FAM	5’ TAACATTCATTGTCCCTTACAGAT 3’ 5’ GCAAGGCTATCTAACGATAATTCA 3’	440-450	96°C - 05min; 94°C -45 Sec, 60°C-45 Sec; 72°C -1min; [25 cycles] 72°C -5min
6	D18S386	PET	5’ TACGGAGAATCACTTGGAAC 3’ 5’ TCCATGAAGTAGCTAAGCAG 3’	340-350	96°C - 05min; 94°C -45 Sec, 60°C-45 Sec; 72°C -1min; [25 cycles] 72°C -5min
7	DX1283	FAM	5’ AGTTTAGGAGATTATCAAGCTG 3’ 5’ TCAAAGTGATCGACAACTACTCAGA 3’	300-340	96°C - 05min; 94°C -45 Sec, 58°C-45 Sec; 72°C -1min; [25 cycles] 72°C -5min
8	AMXY	NED	5’ CTGATGGTTGGCCTCAAGCCT 3’ 5’ ATGAGGAAACCAGGGTTCCA 3’	420-440	96°C - 05min; 94°C -45 Sec, 58°C-45 Sec; 72°C -1min; [25 cycles] 72°C -5min
9	DXS996	NED	5’ AAATTCTTGCTTAGGCCACTCTAGG 3’ 5’ AACGTTGTTCTGGATCGTATGCTA 3’	130-168	96°C - 05min; 94°C -45 Sec, 57°C-45 Sec; 72°C -1min; [25 cycles] 72°C -5min
10	SRY	NED	5’ AGTAAAGGCAACGTCCAGGAT 3’ 5’ TTCCGACGAGGTCGATACTTA 3’	248	96°C - 05min; 94°C -45 Sec, 57°C-45 Sec; 72°C -1min; [25 cycles] 72°C -5min

laboratory is listed in table 1, with the expected range of amplicon size and the physical conditions for their amplification by PCR. For each marker tested, normal result as shown in the Figure 1, gives two peaks of equal height and area (one peak for each chromosome present) if the two alleles are of different length. If the two alleles are of the same length (homozygous), the two peaks are superimposed and this means that a ratio cannot be calculated and the marker is uninformative (see Fig. 1, panel A). If the markers of a particular chromosome show three peaks of

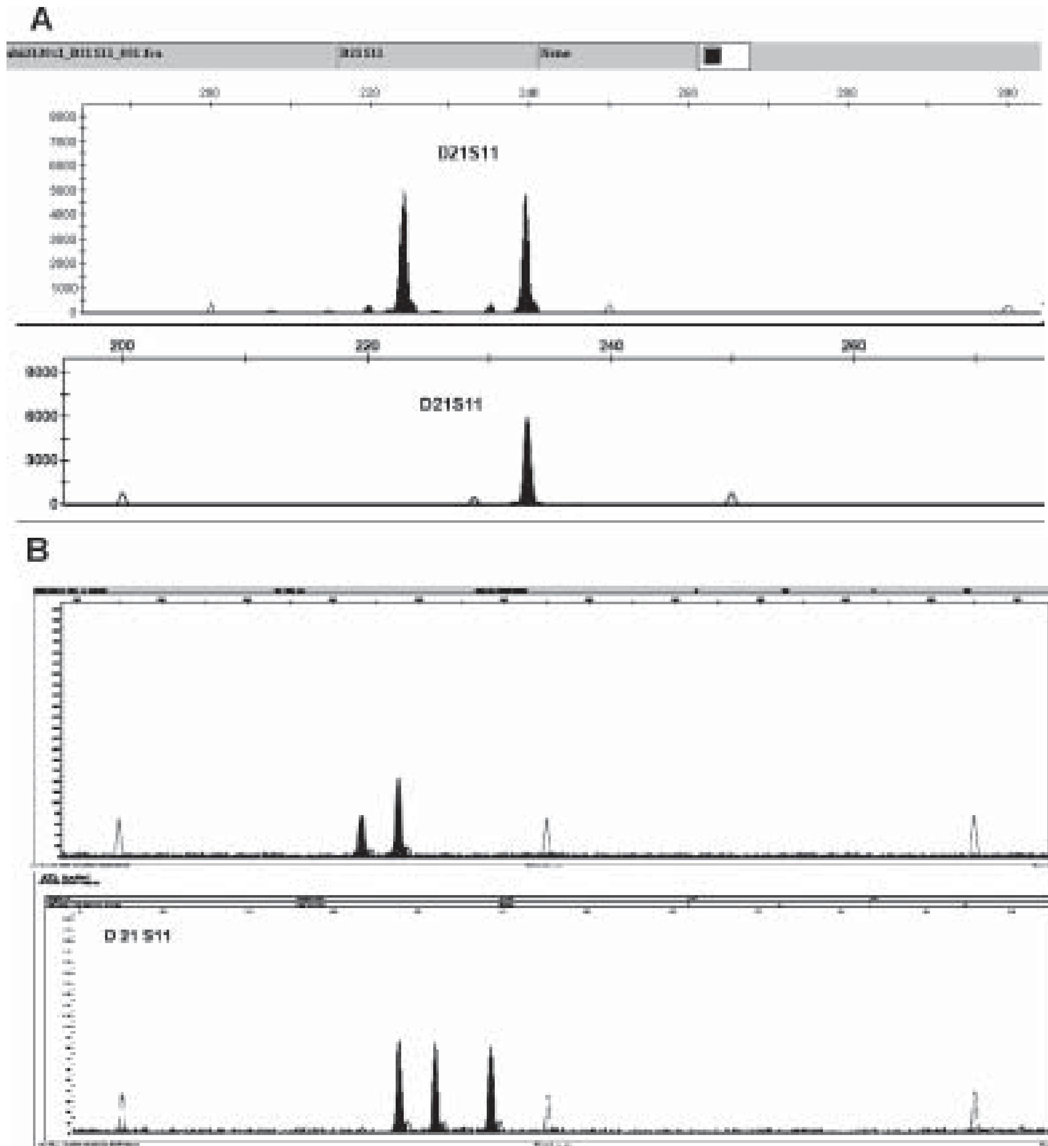

Fig. 1. The result of QF-PCR of normal individuals performed with the microsatellite marker D21S11 for chromosome 21, showing biallelic heterozygous and biallelic homozygous patterns (panel A, top and bottom); and that of individuals with trisomy 21 showing biallelic 2:1 and triallelic patterns (panel B, top and bottom).

equal area, it indicate that there are three different alleles present in a 1:1:1 ratio; and if the marker shows only two peaks but the ratio of the peak area for the two alleles is 2:1, it also indicates the presence of three alleles, two of these alleles having the same length and therefore superimposed, as in the 'normal' example of a homozygote (Fig. 1, panel B).

A number of early publications provide assessment of the of use of small tandem repeat (STR) markers for genetic mapping (Weber 1990; Edwards et al. 1991) and their diagnostic value based on QF-PCR assays for detection of aneuploidies of human chromosomes 21, 18, 13 and the sex-chromosomes X and Y (Mansfield 1993; Pertl et al. 1994, 1996; Adinolfi et al. 1997). The diagnostic advantages of the approach to perform prenatal tests using amniotic fluid and chorionic villi samples, or later from fetal nucleated cells retrieved from peripheral maternal blood or endocervical samples have been demonstrated. The collection of PCR-based polymorphic short tandem repeats genetic markers distributed over the 22 autosomes and the X chromosome during the genetic and physical mapping of the human genome by the Utah marker group has included a majority (85%) of tetranucleotide repeats, because these repeats showed better stability during PCR than the dinucleotide repeats. More than half of these loci (>70%) also had heterozygosity (The Utah Marker Development Group 1995). The use of two or more STR markers in the prenatal diagnosis of aneuploidies for each autosome helps to do away with use of internal non-polymorphic markers. High level of heterozygosity of STR markers employed gives a heterozygous pattern in controls and also multiple alleles pattern in trisomy, which is the easiest one to differentiate. As few as three STR markers per chromosome should be over 99% informative. Multiplex quantitative fluorescent analyses can be performed in about six hours from the collection of the samples and, although targeted to specific abnormalities, they can exclude the presence of the most frequent chromosomal disorders. QF-PCR can also be used to perform prenatal diagnoses on maternal peripheral blood or transcervical cell samples and on preimplantation embryos (Adinolfi et al. 1997). A more complete listing of polymorphic microsatellite markers used in various laboratories testing for chromosomal aneuploidies may be found in Hamilton and Mann (2007).

RESULTS AND DISCUSSION

A few results from our studies for evaluation of the QF-PCR method for diagnosis of trisomies are presented (Fig. 2).

Multiplexing the assay for the diagnosis of aneuploidies of different autosomes and/or sex chromosomes may be performed by combining suitable STR markers in an initial assay. Result of the assay may be further confirmed by introducing the chromosome specific marker for the supernumerary chromosome. Figure 3 shows the results of a multiplex QF-PCR assay positive for trisomy 18.

Simultaneously with prenatal diagnosis of chromosomal aneuploidies the use of sex chromosome specific markers could also be used as control for maternal cell contamination in certain cases. Fetal sexing is usually achieved successfully by including DXS996 and SRY markers in a multiplex reaction and also with amelogenin gene markers for X and Y chromosomes in most cases. Diagnosis of the presence of X and Y chromosomes in the fetal samples often serves as a control for maternal cell contamination in the case of male fetus. Fetal sex determination is not the aim of diagnostic test and the sex of the fetus is seldom disclosed. Amelogenin gene markers for X and Y chromosomes, X chromosomal STRs and Y specific SRY region are all used as markers for sex chromosomes (Table 1). Amelogenin gene serves as a marker for sex determination because the gene is present on the X (*AMEL X*) and the Y (*AMEL Y*) chromosomes of humans and show size differences between these two genes. Thus the gene has been used to differentiate males from females. During our studies results of a few samples showed a false negative result for *AMELY* possibly due to a well known mutation (causing deletion of *AMELY* gene) documented before. Use of additional Y chromosome and X chromosome specific STR markers (SRY and DX 996) in a multiplex PCR with parental controls helped us to identify the chromosomes correctly (Fig. 4). More than one set of markers are suggested for inclusion for the unambiguous identification of sex chromosomes (Jobling et al. 2007; Murphy et al. 2007).

Parental origin of the supernumerary chromosomes and whether they are of pre-zygotic or post zygotic origin may not be deduced from the conventional cytogenetic and other

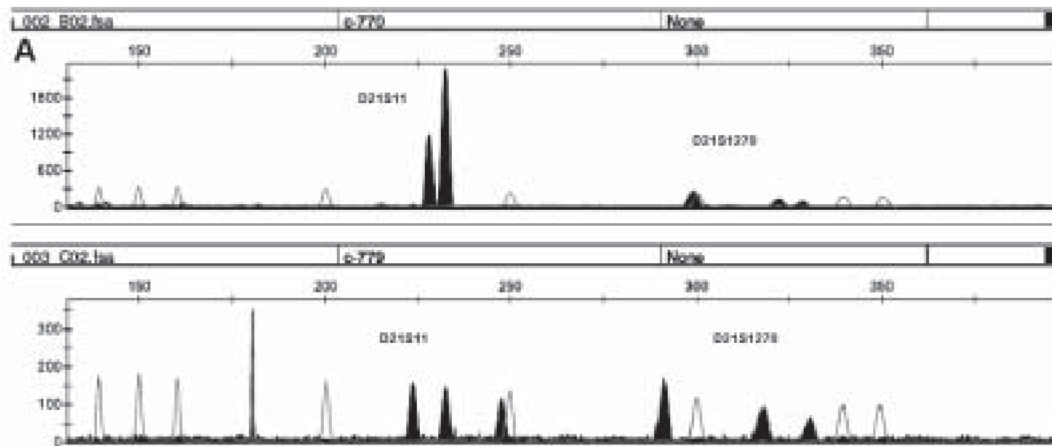

Fig. 2. Prenatal detection of trisomy 21 using D21S11 and D21S1270 markers in QF-PCR assays. D21S11 showed a biallelic 2:1 pattern and D21S1270 showed a triallelic 1:1:1 pattern in the sample shown in panel A. Both markers included in the multiplex PCR assay showed a heterozygous triallelic pattern for chromosome 21 in the sample of panel B.

Fig. 3. Result of a multiplex QF-PCR assay combining markers for chromosome 21 (D21S11 & D21S1270) and chromosome 18 (D18S386) indicating trisomy 18.

analytical techniques. The chromosomal polymorphic STR markers for the detection of major chromosomal aneuploidies can also be used to determine the parent of origin of the supernumerary chromosomes if parental samples are analysed simultaneously for prenatal and postnatal diagnosis of aneuploidies of chromosomes 13, 18, 21, X and Y. The parental origin of the aneuploidy and determination of origin of non-disjunction in meiosis I or II can reliably be evaluated by means of QF-PCR in most cases. Since the quantitative analysis of the PCR product peaks is based on evaluation of the peak height and the peak area, samples with aneuploidy show the peak patterns as either 3 peaks with approximately 1:1:1 height (area) ratio or 2 peaks with 2:1 height (area) ratio. Meiotic origin of the aneupoidy may be inferred by the use of centromeric (most proximal) markers for example, D21S11 and D21S1414 (21q21). The heterozygous pattern observed in the parental sample if found retained in the trisomic offspring indicate an error during meiosis I that occurred in that parent. If the pattern is reduced to homozygous condition in the offspring (with trisomy) it indicates a meiosis II error or an error in a post-zygotic mitosis (Antonarakis 1991). The comparison of the length of PCR fragments in father and mother with that of the progeny would reveal the origin of the extra chromosome. The XXY genotype may occur if the haploid oocyte is fertilized either with one diploid sperm, where the non-disjunction occurred in meiosis I, or with two haploid sperms with X and Y chromosome. Except when alleles of the STRs used for analysis are found in common, in both parents making them uninformative, prediction is possible in most cases. Further addition of highly informative markers decreases the proportion of non-informative families. However, the misinterpretation of

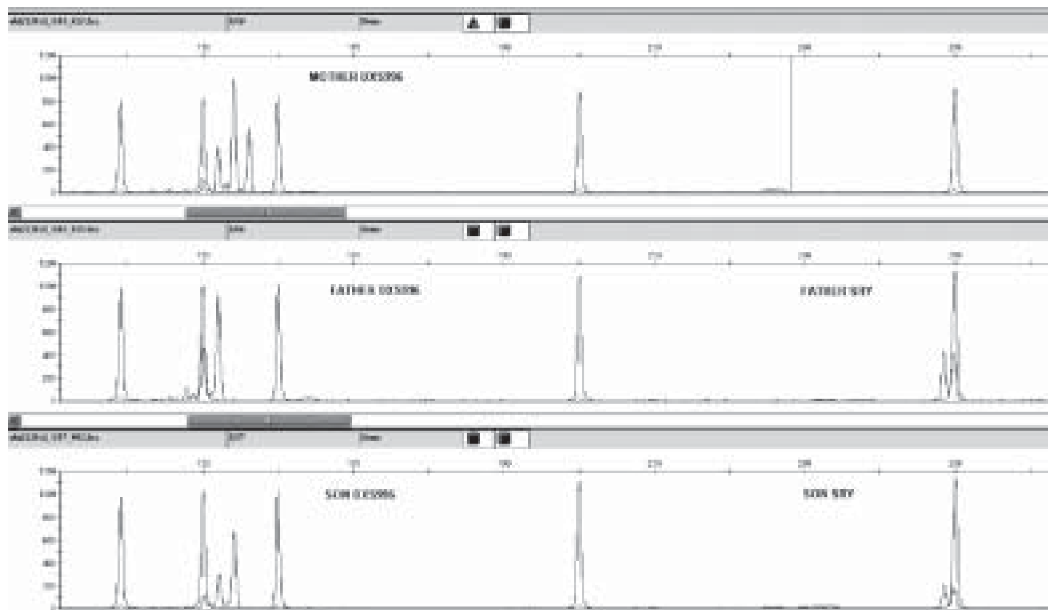

Fig. 4. Alleles corresponding to sex chromosomes detected in multiplex QF-PCR using the polymorphic DX996 (STR) marker and the Y specific SRY marker, in a family; indicating XX of the female and XY of the male chromosome complements.

meiotic origin due to crossing over between the regions containing the marker loci cannot be ruled out. In general, the highly polymorphic markers near the centromere indicate the stage of meiosis in which the disjunction occurred and the homozygosity for all polymor-phic markers in the long arm, as in the case of chromosome 21, indicates post zygotic mitotic non-disjunction (Antonorakis et al. 2004; Machatkova et al. 2005).

In high risk pregnancies detected by ultra-sound markers indicative of Down syndrome or where a serum screen risk is found, rapid confir-mation or exclusion of Down's syndrome help to allow decisions to be made about pregnancy management. The identification of Patau syndro-me (trisomy 13), Edward syndrome (trisomy 18) and sex chromosome aneuploidy could be made using additional probes for chromosomes 13, 18 and the sex chromosomes. These tests could be performed within a few hours. Though the tech-nique is labour-intensive and makes use of expen-sive fluorescent probes, increasing demand for rapid testing might make it viable as an efficient method for the detection of chromosome copy number. Further, multi-plexing the PCRs for the markers makes the test rapid, more efficient and cheap. One or more multiplex may be used to incorporate at least three markers on each of chromosomes 13, 18, 21, and the sex chromosomes (Cirigliano et al. 2001, 2002, 2004). QF-PCR is an efficient test for diagnosis of aneuploidy compared to the alternatives such as FISH and the standard method of karyotyping with cytogenetic techniques. FISH is a more labour-intensive method and there is no diffe-rence between the FISH and QF-PCR techni-ques in either the success rate or the quality of the information obtained. Another advantage of QF-PCR is that, if necessary, it can positively identify samples by analysis of maternal blood, which is not possible by FISH. We can also detect any maternal cell contamination of the sample.

However, there are also a few problems and risk involved in the method of diagnosis. Using QF-PCR, often it may not be possible to detect mosaicism for an abnormal cell line (when it is low level (that is, <15 – 20% abnormal cells). QF-PCR is also not a reliable method for the detection of deletions. Rarely, it is also possible that the samples may be uninformative for all the markers on one or more chromosomes tested for. Such cases may be tested by alternative methods like

FISH or karyotyping. Delayed results may be caused by ambiguous results necessitating further investigation. Stutter bands produced during amplification, mostly of dinucleotide repeat markers makes allele discrimination and quantification sometimes difficult. This may be overcome with the choice of more robust tri- or tetra- nucleotide tandem repeat sequence markers, though. Abnormal results involving maternal cell contamination may pose problems. The sample identity in such cases need to be confirmed by testing maternal DNA sample, repeating the original assay or by interphase FISH. Abnormal markers that are flanked by normal markers may also result due to genomic copy number variations (CNVs). The abnormal and normal results should be detailed in the report and parental sample analysis may be performed to determine the significance of the results Several unexpected findings have been encountered and characterized in studies that have been conducted on larger samples of consecutive patients (Donaghue et al. 2003; Cyrigliano et al. 2002, 2004; Van Zwieten et al. 2005; Hamilton and Mann 2008)

Confounding of prenatal diagnostic testing may occur due to a limited quantity of the sample and its uncertain quality, due to mosaicism in the fetus and through maternal cell contamination (MCC). According to the Clinical Molecular Genetics Society (CMGS) of UK, the question of maternal cell contamination should be addressed by following a few practical guidelines that can improve the quality of diagnosis. Recommended practices may be implemented to ensure quality of diagnostic services. Misdiagnosis resulting from potential contamination of maternal cells in both CVS and amniotic fluid samples should be avoided by using appropriate controls. CVS should be dissected carefully to remove maternal cells deciduas whereas amniotic fluid that is often contaminated with maternal blood staining can be identified, by independently testing maternal blood samples for all the markers (Allen et al. 2008). It is recommended to exclude MCC completely to avoid compromising the validity of the results. A culture of CVS or amniotic fluid derived foetal cells can serve as a repository in case of insufficient sample poses a problem.

CONCLUSIONS

QF-PCR is a rapid, simple and accurate prenatal diagnostic test proved to be efficient and reliable in detecting numerical disorders of autosomes as well as sex chromosomes. For most of the cases it can detect the clinically significant numerical abnormalities of chromosomes X, Y, 21, 18 and 13. It may be useful as a preliminary tool to reduce parental anxiety within a short duration (of approximately 24 hours), and to accelerate therapeutic intervention. However the residual risk involved in the process should be taken note of, suggesting that the testing should be supplemented by conventional cytogenetic analysis to confirm the prenatal diagnoses of normal fetuses. The technique is highly suited for automation and makes it feasible, high throughput analysis of samples at a lower cost. Samples need to be analysed using several selected robust STR markers together with SRY for fetal sex chromosomal analysis. Larger studies have shown that results compare with those obtained by conventional cytogenetic analysis with several advantages. The QF-PCR method has particular significance for countries where conventional cytogenetics is unavailable due to its high cost and lack of skilled manpower, and where it may be used as the only prenatal diagnostic test.

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