Original Article

Two Cases of Klinefelter Syndrome Identified by Quantitative Fluorescence PCR (QF-PCR) Method

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ABSTRACT: Chromosomal aneuploidy is a common cause of genetic abnormality in humans and leading cause of pregnancy loss and congenital birth defects. Pregnancies with chromosomal aneuploidies that survive to term include trisomy 13, 18, and 21 as well as sex chromosome anuploidies with an extra X of Y chromosome. Cytogenetic analysis of metaphase chromosome either by G-banding or fluorescent *in situ* hybridization (FISH) have been the standard methods for identifying aneuploidies and balanced translocation. However 1-2 weeks are required for the completion of the test as the fetal cells require several days of *in vitro* culture. Quantitative fluorescence PCR (QF-PCR) offers a suitable alternative for the diagnosis of chromosomal aneuploides thereby reducing the need of cell culture. QF-PCR analysis includes amplification of chromosome specific short tandem repeats (STRs) and non-polymorphic markers followed by capillary electrophoresis. The main advantage of QF-PCR is its speed, accuracy, ease of automation and allow large number of samples to be investigated at a time. This study reports the successful identification of two cases of Klinefelter syndrome from DNA extracted from peripheral blood and amniotic fluid.

KEYWORDS: aneuploidy, autosome, QF-PCR, Klinefelter Syndrome, trisomy.

INTRODUCTION

Klinefelter syndrome (KS) is a genetic disorder in which there is addition of at least one extra X chromosome to a normal male karyotype (46,XY). The most common form of KS has a 47,XXY karyotype, with a prevalence of 1 in 500 men.[1] Some people with KS have more than one extra sex chromosome in each cell (e.g. 48,XXXY, 48,XXYY or 49,XXXXY), although they are much less frequent. These conditions, which are often called variants of KS, tend to cause more severe symptoms than classic KS.[2] These numerical chromosome abnormalities are acquired through non-disjunction during either maternal or paternal gametogenesis. Males affected with KS may present with a variety of age related symptoms. During the first few years of life, most XXY males do not show any obvious differences from typical male infants. Some may have slightly weaker muscle, small phallus or cryptorchidism.[3] After the age of 5 years, boys with KS may be slightly taller and present with learning disabilities, language delay, or behavioral problems.[4] The pubertal development in adolescent boys may be disrupted as they produce less testosterone compared to non-KS boys, and may be presented with gynecomastia and small testes.[5] The most frequent problem in adult KS is azoospermia and infertility.[6] Phenotypes are typically characterized by tall stature, narrow shoulders, broad hips, sparse body hairs and often evaluated for infertility and breast malignancy.[7] Males with poly-X Klinefelter syndrome may have more pronounced symptoms than in males with KS.[8]

The most common method for the detection of chromosomal aneuploidies and balanced translocation is the metaphase karyotype analysis by cytogenetic techniques.[9] However, the sensitivity of karyotyping depends on the number of cells examined in a particular culture and several days are required for the completion of the test. Moreover, random distribution of chromosomes may hinder the accuracy of band

comparison, highly dependent upon the experience and skill of the analyst and ineffective in detecting small translocation or microdeletions. In addition to karyotype analysis, fluorescent *in situ* hybridization (FISH) has been introduced during the past two decades. This method is more sensitive than karyotype analysis because, it can be used on both dividing and non-dividing cells, and based on the painting of whole or part of the chromosome with fluorescently labeled probes.[10] However, FISH is labor-intensive, not easily applicable to a large number of samples and cannot be automated.

In recent years, quantitative fluorescence polymerase chain reaction (QF-PCR) has contributed greatly in the diagnosis of chromosomal aneuploidies directly from blood, amniotic fluid or CVS without the need of cell harvesting with a rapid turnaround time. This method relies on the amplification of polymorphic short tandem repeats (STRs) specific to chromosome 13, 18, 21 and sex chromosomes. The PCR products are then analyzed on autoamted DNA sequencer. Since the primers are labeled with fluorescent dye, a triallelic peak pattern or diallelic pattern with quantitative difference is observed in cased of chromosomal aneuploidy. Usually, more than one STR markers are used for each single chromosome to achieve informative results on the chromosome copy number. In this study we report the identification of two cased of Klinefelter syndrome by QF-PCR method.

MATERIALS AND METHODS

Case presentation
Two patients apparently suffering from azoospermia was referred to the laboratory for chromosomal aneuploidy check. They were 34 and 52 years old respectively with a male phenotype.The first one had very small testis and the second one had feminine voice with a bulky body stature. Both of them had a medical history of infertility.

Sample and DNA extraction
Approximately 3.0 mL peripheral blood samples were collected in an EDTA tube, from both the patients. Genomic DNA was extracted by using QIAmp DNA blood mini kit (Qiagen, Genmany, Cat No. 51104). Extracted DNA was quantified by Quantus™ fluorometer (Promega Corporation, USA).

PCR amplification and typing
About 3-5 ng of template DNA was used for each PCR amplification process. For PCR amplification, a total of 26 chromosome specific markers were amplified in a multiplex fashion using Devyser Compact v3 kit (Devyser AB, Instrumentvägen 19 SE-126 53 Hägersten Sweden). The PCR products were separated on 3500 Dx Genetic Analyzer (Applied Biosystems, USA) using POP-7 polymer and data collection software v1.0. Peak sizing and genotype assignments were done by GeneMapper v4.1 software. Quantification was achieved by calculating the ratio of the specific peak areas of the respective alleles.

Table 1. Biological characteristics of DNA markers used for the detection of chromosomal aneuploidy.

| ID	Marker	Chromosome location	Marker type	Chromosomal occurrence	Marker size range (bp)	Dye color
13A	D13S742	13q12.12	Polymorphic STR sequence	Chr 13	222-334	Green
13B	D13S634	13q21.32-q21.33	Polymorphic STR sequence	Chr 13	365-435	Blue
13C	D13S628	13q31.1	Polymorphic STR sequence	Chr 13	420-475	Yellow
13D	D13S305	13q13.1	Polymorphic STR sequence	Chr 13	435-505	Green
13K	D13S1492	13q21.1	Polymorphic STR sequence	Chr 13	100-175	Red
18B	D18S978	18q12.3	Polymorphic STR sequence	Chr 18	195-230	Yellow
18C	D18S535	18q12.3	Polymorphic STR sequence	Chr 18	300-350	Blue
18D	D18S386	18q22.1	Polymorphic STR sequence	Chr 18	338-430	Green
18J	D18S976	18p11.31	Polymorphic STR sequence	Chr 18	440-495	Red
18M	GATA178F11	18p11.32	Polymorphic STR sequence	Chr 18	350-410	Yellow
21A	D21S1435	21q21.3	Polymorphic STR sequence	Chr 21	150-208	Blue
21B	D21S11	21q21.1	Polymorphic STR sequence	Chr 21	215-29	Blue
21C	D21S411	21q22.3	Polymorphic STR sequence	Chr 21	245-345	Yellow
21D	D21S1444	21q22.13	Polymorphic STR sequence	Chr 21	440-495	Blue
21H	D21S442	21q21.3	Polymorphic STR sequence	Chr 21	362-420	Red
21I	D21S1437	21q21.1	Polymorphic STR sequence	Chr 21	105-152	Yellow
X1	DXS1187	Xq26.2	Polymorphic STR sequence	Chr X	120-170	Green
X3	XHPRT	Xq26.2-q26.3	Polymorphic STR sequence	Chr X	265-308	Red
X9	DXS2390	Xq22.33-Xq11.32	Polymorphic STR sequence	Chr X	312-357	Red
XY2	DXY267	Xq21.31, Yp11.31	Polymorphic STR sequence	Chr X & Y	175-217	Green
XY3	DXY218	Xp22.33, Yp11.32	Polymorphic STR sequence	Chr X & Y	215-260	Red
AMELXY	AMELX, AMELY	Xp22.2, Yp11.2	Non-polymorphic sequence	Chr X & Y	X=104, Y=110	Blue
SRY	SRY	Yp11.31	Non-polymorphic sequence	SRY Gene	236	Yellow
ZFXY	ZFX, ZFY	Xp22.11, Yp11.31	Non-polymorphic sequences	Chr X & Y	157-166	Yellow
T1	-	Xq13, 7q34	Non-polymorphic Sequence	Chr X & 7	Chr7 =181, X=201	Red
T3	-	Xq21.1, 3p24.2	Non-polymorphic Sequence	Chr X & 3	Chr3=133, X=137	Blue
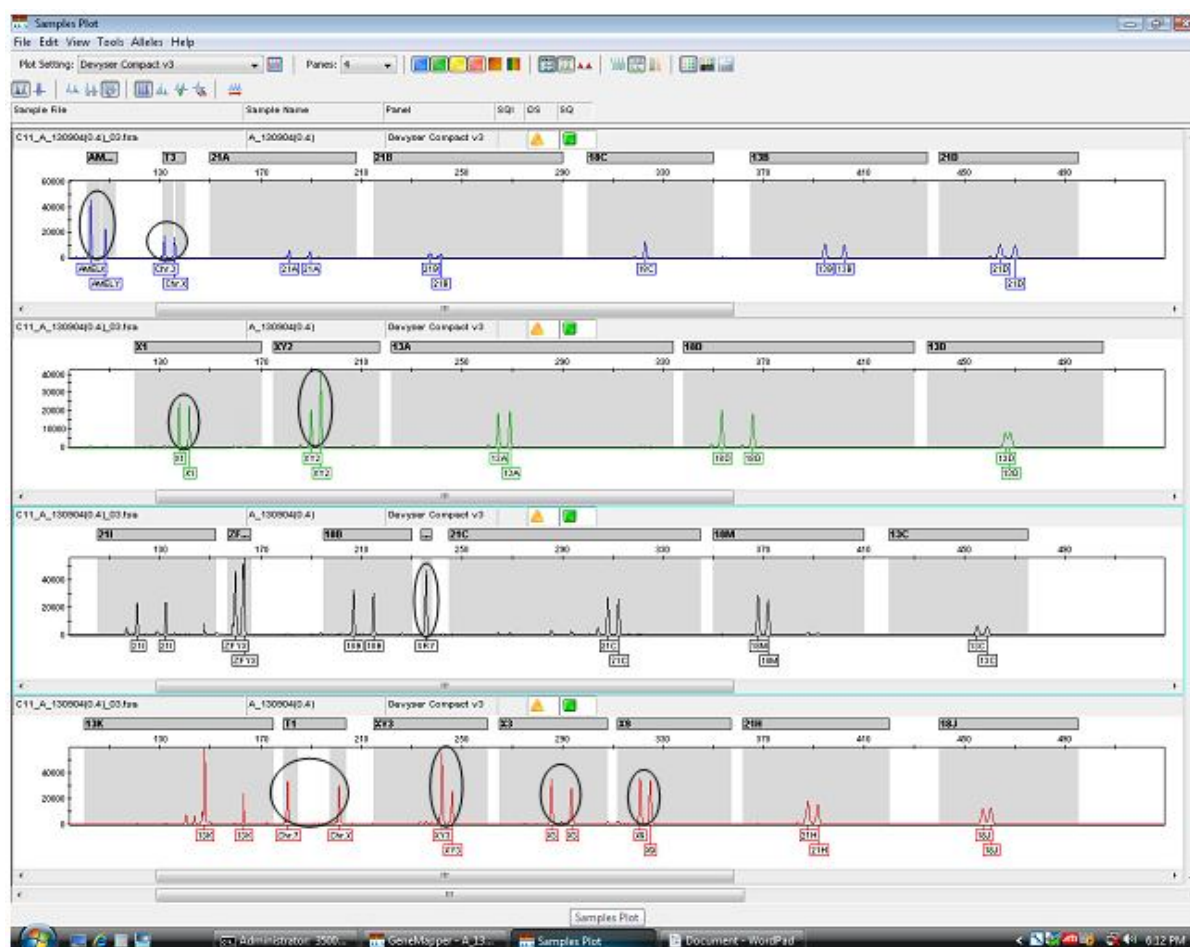
Figure 1. Elctropherogram showing the chromosomal aneuploidy of patient 1 generated by capillary electrophoresis.

RESULTS AND DISCUSSION

The QF-PCR analysis of the two studied samples revealed that both the patient had one extra X chromosome, typically characteristics of Klinefelter syndrome. The results are presented in Figure 1 & 2 respectively. The analysis was conducted using a total of 26 STR markers presented in Table 1. For the detection of numerical abnormalities, chromosome specific polymorphic STR markers were chosen. STRs or short tandem repeats are small DNA sequences of 2-7 bp in length, repeated in tandem and account for 3% of the human genome.[11] They are highly polymorphic and occur on average in every 10,000 nucleotides. Due to their small dimension, low mutation rate, genome wide distribution and high level of polymorphism, these markers are intensely used as important genetic markers in personal identification, parentage testing and population genetics study.

For chromosome 13, five STR markers such as, 13A, 13B, 13C, 13D and 13K were selected. STRs vary in size between subjects, depending on the number of repeat units present on each chromosome. Since the PCR primes are labeled with fluorescent dyes, each PCR product is visualized as specific peaks in the elctropherogram after capillary electrophoresis. Quantitation can be achieved by calculating the ratio of the specific peak areas of the respective STR using

an automated DNA sequencer. DNA amplified from normal subjects who are heterozygous for a specific STR marker is expected to show two peaks of same area with a ratio of approximately 1:1. The subjects who are homozygous or monosomic will display only one peak in a specific marker and is considered to be uninformative. DNA amplified from subjects who are trisomic will exhibit either three peaks with similar area (area ratio 1:1:1) or two peaks, one of them twice as large as the other (area ratio 1:2 or 2:1).

The analytical output for both the samples under study is presented in Figure 1 & 2. No numerical abnormality for chromosome 13 was detected as marker 13A, 13B, 13C, 13D and 13K yielded only one peak or two peaks of the same height. STR markers analyzed for chromosome 18 (e.g. 18B, 18C, 18D, 18J & 18M) and chromosome 21 (e.g. 21A, 21B, 21C, 21D, 21H & 21I) also did not show any triallelic pattern or peak imbalance in any of the samples (Figure 1 & 2). It therefore confirms that normal diploid complements exist for chromosome 13, 18 and 21 in both the patients.

Marker X1, X3 and X9 are polymorphic STR markers present on X chromosome only. It therefore should produce single peak for males who has only one X chromosome. For females it should produced single peak for homozygotic female when the STR marker has
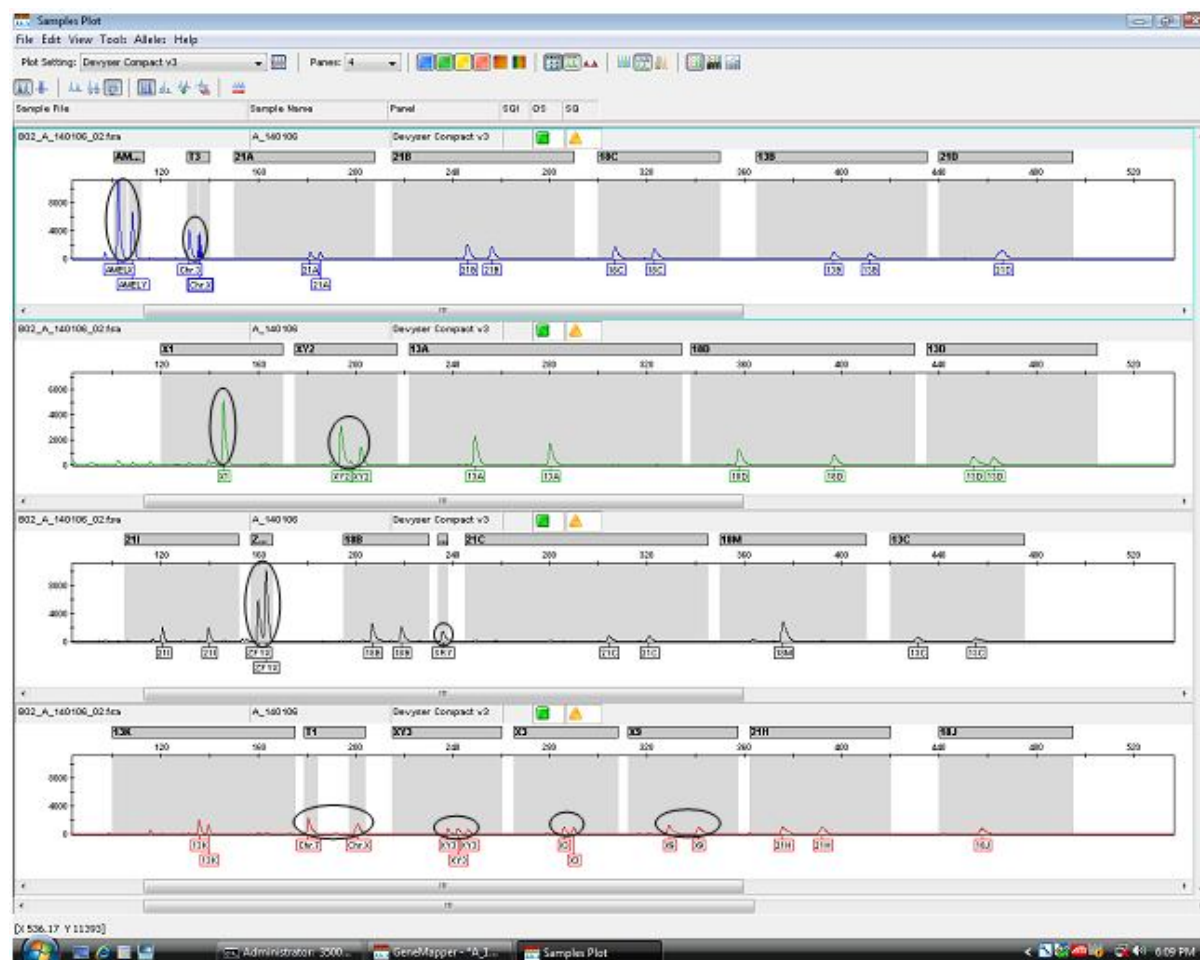
Figure 2. Elctropherogram showing the chromosomal aneuploidy of patient 2 generated by capillary electrophoresis.

same number of repeats on both the chromosome and two peaks for heterozygotic female when the number of repeats is different. Since the chromosomal location and the primer binding sites are different for these markers, it is highly unlikely that they will have same number of repeats from both the chromosomes for all the three markers. In our study, we obtained two peaks from all the markers for patient-1 (Figure 1) and single peak for marker X1 and two peaks for marker X3 and X9 from patient-2 (Figure 2). This indicates the presence of more than one X chromosome in both the patients (Figure 1 & 2).

In order to check the number of X and Y chromosome two other polymorphic STR markers such as, XY2 and XY3 were selected which is present on both X and Y chromosome. For normal males these markers should produce single peak, whereas, in homozygotic female they should produce single peak. If there is a presence of any extra X or Y chromosome they should produce either triallelic pattern or two peaks with an imbalance of 1:2 or 2:1. For patient-1 (Figure 1) a peak imbalance of 1:2 and 2:1 was observed at XY2 and XY3 markers respectively. On the other hand a peak imbalance of 2:1 and a triallelic pattern was observed for XY2 and XY3 markers respectively (Figure 2). These findings clearly indicate that both the

individuals are having either one extra X or Y chromosome.

AMELXY is another marker used to for gender identification. The marker is selected from amelogenin gene which encodes a protein present in tooth enamel. The X homologue has a 6 bp deletion in intron 1 of the amelogenin gene. Two X chromosome in females produces PCR products of same length and gives only one peak. In males on the other hand, the PCR product from Y chromosome is 6 bp larger and the PCR product therefore produces two peaks of equal height. If there is any sex chromosome aneuploidy the corresponding peak area will be doubled for any extra X or Y chromosome. In both the study subjects the ratio of AMELX and AMELY was found to be approximately 2:1 which further confirms the presence of one extra X chromosome (Figure 1 & 2).

The ZFXY is a no-polymorphic (no-STR) marker present on both X and Y chromosomes. The relative amount of ZFX and ZFY products for individuals with 47, XXY or 47,XYY would expected to produce two peaks with a ration of 2:1 or 1:2. However, It is not possible to determine which allele represent X or Y chromosome. These markers are used to assess the total number of sex chromosome when informative. In both Figure 1 and Figure 2 the ZFXY produced two

peaks with a ratio of approximately 1:2 indicating the presence of either one more X or Y chromosome. The SRY marker was chosen from the SRY (Sex determining region of Y chromosome) gene which is present only on Y chromosome. The amplified sequence of SRY gene was a non polymorphic sequence and should only be amplified in males or if there is a presence of Y chromosome. In both patients we found a SRY positive peak indicating the presence of Y chromosome.

The T1 and T3 markers are non-polymorphic X chromosome counting markers that are used to determine the number of X chromosome. The X chromosome counting markers define sequences present on the X chromosome and an autosomal chromosome that are amplified using identical primers. For T1 the PCR reaction amplifies a 201 bp sequence from X chromosome and a 181 bp sequence from chromosome 7. The T3 on the other hand amplifies a 137 bp sequence from X chromosome and a 133 bp sequence from chromosome 3. The amplified marker fragments are separated according to their size and the copy number of X chromosome is determined by the fragment area ratio calculation. In a normal female X chromosome counting marker an area ratio of 1:1 is expected. In normal males and females with monosomy of X a 2:1 ratio is expected. In both the study samples the T1 and T3 markers produced a peak area ratio of 2:1 (Figure 1 and Figure 2) indication the presence of two X chromosomes.

Considering the findings described above it can be concluded that both the tested individuals were having Klinefelter syndrome with a karyotype of 47,XXY. Among the other chromosomes which were checked for any numerical abnormality, such as chromosome 13, 18 and 21, a normal diploid complement was identified. The presence of SRY gene in both the patients confirms that they were indeed Kilinefelter male. The extra X chromosomes in patients with Klinefelter syndrome interferes with male sexual developments, often preventing the testes functioning normally and reducing the levels of testosterone. In both the study subjects the clinical presentations as described in the materials and methods section is consistent and thereby confirms the diagnosis of a Klinefelter male.

In addition to the diagnostic efficiency the QF-PCR method used in this study offers a suitable alternative of standard karyotyping with cytogenetic methods or fluorescent *in situ* hybridization (FISH). FISH is more labor-intensive compared to QF-PCR technique in terms of success rate or quality of information that is achieved. The polymorphic STR markers used for the detection of major chromosomal aneuploidies can also be used to determine the parent-of-origin of the supernumerary chromosome if parental samples are analyzed simultaneously. Furthermore, this method offers quicker turneround time without the need of cell culturing with the capability of detecting maternal cell contamination in amniotic fluid or chorionic villus samples.

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