Segmental duplication – quantitative fluorescent-polymerase chain reaction: An approach for the diagnosis of down syndrome in India

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Abstract

Objectives: Early detection of high risk pregnancies of down syndrome (DS) is the main target of offering prenatal diagnosis. Segmental Duplication Quantitative Fluorescent PCR (SD-QF-PCR) can be used as an alternative method for prenatal diagnosis of DS. SD-QF-PCR involves segmental duplications sequences between the test and control chromosomes to detect aneuploidies.

Materials and Method: Segmental duplication are two similar sequences with different fragment lengths, located on two different chromosomes. When these segmental duplication regions are amplified, the peak ratio between the two different chromosomes remains 0.9 to 1.1 and the trisomy 21 results in the ratio of 1.4 to 1.6. In this study, we applied SD-QF-PCR method to detect the presence of trisomy 21 in sixty age-matched controls and sixty DS samples. The PCR amplification of segmental duplication regions is done using a single pair of fluorescently labelled primers, the peak ratio between the two different chromosome regions are evaluated.

Results: All sixty control samples showed the peaks to range from 0.9 to 1.1 which was suggestive of normal samples while peaks of sixty-five DS samples range from 1.4 to 1.6 which suggested the presence of trisomy 21. Conclusion: Segmental Duplication Quantitative Fluorescent PCR is a sensitive and rapid aneuploidy detection technique hence can be used as a standalone test to detect trisomy 21 as well as other aneuploidies.

Keywords: SD-QF-PCR, aneuploidies, trisomy 21, prenatal diagnosis

Introduction

The trisomy 21 is the main cause of down syndrome (DS) and it is associated with various others clinical phenotypes like alzheimer’s diseases, congenital heart diseases, cancers, Hirschsprung’s disease, Leukemia’s, epilepsy, sleep disorder, infertility related issues and a various nutrient deficiencies. The incidence of trisomy 21 is 1 in 1000 live birth however it differs among ethnic groups(1). According to National Down Syndrome Society survey, the life expectancy for DS individuals is 55 years(2,3,4). DS are associated with various characteristics facial features like Hypotonia, craniofacial abnormality, flat facial profile, excessive skin at the nape of neck, hypotonia, hyper flexibility of joints, dysplasia of pelvis, anomalous ears, dysplasia of mid phalanx of fifth finger and transverse palmer crease (simian crease) in early infancy(4,5). Besides these, the other common features includes: an upward slant to the eye, flat nasal bridge, short neck, abnormally shaped ears and white spots on the iris of the eye (called Brushfield spots(6)). Most of these patients have mild to moderate intellectual disability. DS child can be prevented by offering a prenatal diagnosis to high-risk pregnancies. However, the sampling methods, chorionic villus sampling and amniocentesis are associated with 0.5 -1% risk of miscarriage(7). Soft markers like small or absence of nasal bone, increased thickness of nuchal fold and presence of large ventricles are used to detect the risk of trisomy by ultrasound at 12 to 24 weeks of gestation(6,8). The cytogenic analysis method is widely used as “gold standard” method for offering prenatal diagnosis. However, rapid aneuploidy testing methods like fluorescent in situ hybridization (FISH), quantitative fluorescence-polymerase chain reaction (QF-PCR), and multiplex probe ligation assay (MLPA) are also routinely used for a prenatal diagnosis in the laboratory(4). A novel technique, segmental duplication quantitative fluorescent PCR was established by Kong et. al in 2014(9) which involves segmental duplications sequences between the test and control chromosomes to detect aneuploidies. Segmental duplications are two similar sequences with different fragment lengths, located on two different chromosomes. The method involves amplifying segmental duplication regions. When these sequences are amplified using a single pair of fluorescently labelled primers, the peak ratio between the two different chromosomes remains 0.9 to 1.1 and the trisomy 21 results in the ratio of 1.4 to 1.6(9,10).
Materials and Methods

The study included sixty DS patients confirmed by karyotype (Figure 3) and sixty control samples after obtaining informed consent. Two mL of peripheral venous blood was collected in EDTA from SGPGIMS, Lucknow, India. The study was approved by the institutional ethics committee (SGPGIMS, Lucknow, India). The work has been carried out in accordance with the code of Ethics of the World Medical association (Declaration of Helsinki,1975 revised in 2000) for experiments in humans. Genomic DNA isolation was done using standard Phenol-Chloroform method followed by PCR amplification using primers obtained from elsewhere (Muthuswamy et al., 2016). The PCR conditions include initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of 30 seconds at 95 °C, 30 seconds at 60 °C, and 30 seconds at 72 °C and a final extension step at 72 °C for 10 minutes. Amplified PCR products (2 µL) were denatured with 8 µL HiDi and 0.5 µL LIZ at 95 °C for 5 minutes and loaded onto the genetic analyser (ABI 310 Genetic Analyzer, Applied Biosystems). On the basis of the area acquired by the peak, the relative peak signal ratios were calculated. The expected value for a normal and trisomic sample are 0.9 to 1.1 and 1.4 to 1.6 respectively.

Results

SD-QF-PCR confirms all sixty DS patients to be positive for trisomy 21. Figure 1A shows the resulting peak in the case of euploids the expected value was between 0.9 to 1.1 while for the trisomy the value changes to 1.4 to 1.6 confirms the presence of an extra region. The figure 1B shows the expected value of the ratio for euploid, monosomy and trisomy. The figure 2A shows the electropherogram obtained after SD-QF-PCR for euploid samples showing normal allele ratio for both the markers, 21/11 and 21/6. The figure 2B shows the electropherogram obtained after SD-QF-PCR for trisomy patient samples showing values between 1.4 to 1.6 for markers 21/11 and 21/6 respectively confirming the presence of DS.

Discussion

The study aimed to confirm the utility of SD-QF-PCR as an alternative method for postnatal diagnosis of DS as well as can be employed for prenatal diagnosis also. We have recruited sixty age-matched controls and sixty DS samples and checked these samples for the presence of trisomy by the amplification of segmental duplication regions using a single pair of fluorescently labelled primers. The peak ratio between the two different chromosome regions were evaluated and for euploid the expected value was found to be in between 0.9 to 1.1 and the expected value for trisomy 21 cases was found to be in between 1.4 to 1.6. All the samples were correctly diagnosed by using the SD-QF-PCR method and the informativeness of the markers was found to be 100%. SD-QF-PCR method offers various advantages over various molecular based methods for both prenatal and post natal diagnosis of DS. Cytogenetic analysis of metaphase chromosomes is performed on metaphase stage fetal cells on amniotes creating unique banding patterns on the chromosomes. However, cytogenetic analysis is a time-consuming process and the detection of trisomy 21 requires at least 2-3 days. On the other hand, SD-QF-PCR is a rapid and efficient method for the detection of trisomy 21 in a single day.

Figure 1. A shows the ratios of resulting peak that will be obtained after normal or DS individual. B: shows ratios for normal and trisomy.

Figure 2. Results of SD-QF-PCR. A) Normal individuals showing all normal sized alleles. B)DS patients showing 1:2 peak ratio for markers 21/11 and 21/6 respectively.

Figure 3. Chromosome as visualized on conventional karyotyping. A) Normal chromosome 21 pair, B) Normal chromosome 22 pair, C) Trisomy 21 showing presence of extra allele.
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<th>Advantages</th>
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<tr>
<td>Cytogenetics Analysis</td>
<td>Giemsa banding (G-banding) is performed on fetal cells at metaphase stage on amniocytes (grown in vitro) or CVS</td>
<td>Suitable for low income countries where physician can be presumed to have acquired a high level of diagnostic skill in the absence of laboratory services</td>
<td>Time consuming, Resoluiion of special importance for the detection of structural abnormalities may be quite low as the spontaneous dividing cells are more condensed than those obtained after cell culture in vitro. In CVS, occurrence of confined placental mosaicism and occurrence of aberrant cells that do not represent the status of fetus. Chances of giving a false positive and false negative result.</td>
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<td>Fish (Fluorescence in situ Hybridization)</td>
<td>FISH involves hybridization of selected chromosome specific DNA sequences that have been labeled with fluorescent dye to chromosome preparation. The fluorescently labeled sequences stick to corresponding DNA of chromosome and can be visualized under microscope.</td>
<td>As it uses smaller probes thus the signals appears to be more distinct as dots, It uses higher number of interphase nuclei for analysis, so the problem of any suspected mosaicism is resolved</td>
<td>Sometimes diffused signals are obtained because it uses chromosome at interphase stage which appears less condense than those of metaphase. Time consuming since it involves preparation of slides, fluorescent microscopy and spot counting (~30min per sample is expected). Maternal and fetal XX is not distinguished by FISH</td>
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<td>Qf-Pcr (Quantitative Fluorescent-Polymerase Chain Reaction)</td>
<td>Involves amplification and detection of STR using fluorescently labeled primers. The product is thus visualized and quantified as peaks areas of respective length using an automated DNA sequencer with Gene Scan software.</td>
<td>Highly reliable and reproducible, Chances of getting false negative and false positive cases are rare. Faster approach as it can give the diagnosis within 24hours</td>
<td>Poses a challenge in the case of mosaicism. While testing sex chromosome abnormalities samples from normal XX female may show homozygous QF-PCR pattern indistinguishable from those produced by sample with single X as in Turner syndrome.</td>
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<td>Paralogous Sequence Quantification (Pq)</td>
<td>A PCR based method for detection of targeted chromosome number abnormalities, based on the use of paralogous genes. Paralogous sequences have high degree of sequence identity but accumulate nucleotide substitution in a locus specific manner. These differences are called as paralogous sequence mismatches which can be quantified using pyrosequencing.</td>
<td>The first generation design of test requires 10 separate PCR reaction per sample, which significantly reduces the sample throughput and increases the probability of handling errors. It can handle 30-40 samples in a day and report result in less than 48 hours</td>
<td>Expensive when compared to others. Required a skilled bioinformatics analysis</td>
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<td>Mloa (Multiplex Probe Ligation Assay)</td>
<td>MLA is based on hybridization and PCR method. Divided into 4 phases: DNA denaturation, hybridization of probe to the complementary target sequence, probe ligation and PCR amplification of ligated probe. These amplified products are analysed through capillary electrophoresis.</td>
<td>Very short time for diagnosis(2-4 days) Relatively low costs</td>
<td>Unable to exclude low level placental and true mosaicism.</td>
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<td>Ngs (Next Generation Sequencing)</td>
<td>Clonally amplified DNA templates are sequenced in a massively parallel. It provides a digital quantitative information, in that each sequence read is a countable “sequence tag” representing an individual clonal DNA template or a single DNA molecule</td>
<td>The current time for sample processing, sequencing, and data interpretation in experienced hands is 5 to 8 days.</td>
<td>The cost of sequencing is approximately $700 ~$1000 per sample. Complex data analysis.</td>
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Table 1. Continued.

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<td>Segmental Duplication – Quantitative Fluorescent PCR</td>
<td>Segmental duplications are two similar sequences with different fragment lengths, located on two different chromosomes maintaining the original ratio between the two different chromosomes upon amplification using a single pair of fluorescent primers. The PCR amplified products of different sizes are analyzed through capillary electrophoresis and the trisomy 21 are determined based on the relative dosage between the two chromosomes in a single reaction. This method is called as segmental duplication quantitative fluorescent PCR</td>
<td>Unlike other methods, can easily detect all ploidy levels and maternal contamination. Robust and cheaper than above mentioned methods. Faster approach as the diagnosis can be given in 12 hrs</td>
<td>Mosaicism cannot be detected.</td>
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Consuming method and labor intensive\(^{(9,11)}\). MLPA can also be employed to evaluate copy number of DNA sequences and offers a number of advantages like – simple, cost effective and requires a very short time for giving the diagnosis. MLPA is divided into has four steps: DNA denaturation, hybridization probe ligation, PCR amplification. After PCR amplification, the amplified products are loaded onto the genetic analyser for capillary electrophoresis. Overnight hybridization step in this method makes MLPA as labor intensive. However, MLPA is unable to detect low level placental and mosaicism. The major drawback of MLPA is that it offers mosaicism or maternal cell contamination. MLPA also uses labeled – probes which are quite expensive, thus making this method as expensive\(^{(12,13)}\). The most widely used method for prenatal diagnosis is FISH, which is performed on interphase nuclei, using chromosome specific fluorescently labelled probes. The main drawback of FISH is that it is a low throughput method, involving hybridization of fluorescently labelled chromosome-specific DNA\(^{(14)}\). And also, sometimes diffused signals are seen in the case of interphase chromosome\(^{(14,17)}\). An alternative method, QF –PCR is a self-tuning regulator based markers approach which is present on chromosome 21 and by using these markers we can detect trisomy in 86.67% cases with only two markers and further using more number of markers can increase the reliability of the test. Thus, QF-PCR is a robust, sensitive and an automated technique and can handle a lot of samples at a time. The main advantage of this techniques is that the diagnosis can be given with 12 hours\(^{(9)}\). Non-disjunction of parental origin can also be detected simultaneously. However, QF-PCR fails to detect mosaicism and various ploidy levels. However these problems were overcome by the SD-QF-PCR method, were various ploidy levels and maternal contamination can easily be detected. Thus, SD-QF-PCR is a robust and cheaper than above-mentioned methods. It is much faster approach than all above-mentioned assay as the diagnosis can be given within 12 hours.

The present report confers the utility of segmental duplication QF-PCR for rapid detection of aneuploidies for developing countries like India. SD-QF-PCR can act as a standalone test for detection of DS as well as other ploidy levels as other commercial methods as expensive and time consuming compared to other rapid assays. Furthermore, the present study which was conducted in prenatal samples using genomic DNA from DS patients reported the sensitivity of this technique to be 100%. Similar studies can also be conducted in prenatal samples from the high risk pregnancies for aneuploidies which can further establish this technique as alternative standalone test for prenatal diagnosis of aneuploidies as well.

**Conclusion**

The primary target of prenatal diagnosis is an early detection of high risk pregnancies of DS. The choice about that after prenatal diagnosis of DS whether or not to continue a pregnancy is a complex process as it involves various socio-economic factors. The risk for fetal trisomy can be evaluated on the basis of various factors like prior family history, maternal age, fetal ultrasound markers and biochemical test of maternal serum. Women who are identified as high risk carriers can receive genetics counseling and other additional test like cytogenic analysis and other molecular methods like: FISH, QF-PCR, and MLPA can be employed. As these above mentioned methods offers different disadvantages that we have just discussed in the above section of this paper which was overcome by the novel SD-QF-PCR method. Hence, SD-QF-PCR is an automated, rapid, reliable, sensitive and robust technique and can be used for diagnosis of various ploidy levels in a clinical setup.

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