Segmental Duplication QF-PCR: A Simple and Alternative Method of Rapid Aneuploidy Testing for Developing Country Like India

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Background: Aneuploidy screening is becoming an integral part of routine prenatal screening in developing countries like India, and the need for more cheaper and rapid aneuploidy testing methods are required to relieve the anxiety and financial burden among the high-risk couples. Segmental duplication quantitative fluorescent polymerase chain reaction (SD-QF-PCR) emerged as an alternative aneuploidy diagnostic method. **Methods:** This study was conducted to optimize and assess the utility of SD-QF-PCR in routine prenatal diagnosis to complement existing short tandem repeats (STR) based

QF-PCR. About 50 control samples, 50 Down's syndrome samples, and one each trisomy 18 and Klinefelter samples were studied to optimize the assay. Later, 100 amniotic fluid samples were also studied.

Results and conclusion: The assay was able to successfully identify normal and aneuploidy samples with 100% sensitivity and specificity. The results of amniotic fluid analysis by SD-QF-PCR were in agreement with results of STR-QF-PCR. Observed results qualify SD-QF-PCR as a preliminary aneuploidy diagnosis method. J. Clin. Lab. Anal. 31:e22038, 2017. © 2016 Wiley Periodicals, Inc.

Key words: aneuploidy; Down's syndrome; quantitative fluorescent PCR; rapid aneuploidy testing; segmental duplication; trisomy

INTRODUCTION

Aneuploidy screening is becoming a part of routine obstetrics procedure in developing countries like India. Followed by screening, high-risk couples are counseled for prenatal diagnosis and allowed to decide upon it. For such couples, invasive sampling is done and sent to laboratory for diagnosis.

The diagnosis is carried out by various methods such as traditional karyotyping to different molecular methods such as fluorescence in situ hybridization (FISH), multiplex ligation-dependent probe amplification (MLPA) and QF-PCR (short tandem repeats (STR) based; STR-QF-PCR), and more recently SD-QF-PCR (segmental duplication QF-PCR) is introduced. The couples undergoing prenatal diagnosis are anxious until the day of reporting. The turnaround time for traditional karyotyping is around 2 weeks, which is too long for the patients as well as for the pregnancy with late gestation age. In such situations, molecular methods are robust in providing reports within few hours to a couple of days. Such methods

are FISH, MLPA, and STR-QF-PCR and collectively referred to as rapid aneuploidy testing (RAT) (1). In 2014, a research group from China demonstrated a novel quantitative fluorescent PCR based on segmental duplication regions on two different chromosomes for aneuploidy diagnosis (2). Similarly, in the past, it has been also reported to be accurate in diagnosis of sex chromosomal abnormalities (3).

Among the RAT methods, QF-PCR is considered as more versatile due its ability to check maternal contamination, origin of non-disjunction, and cost of the test (4). In spite of its advantages, the drawback is pertaining to the establishment of the method. Since the method requires information on STR markers heterogeneity for a particular population to be tested,

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it requires a prior population-based study to establish heterozygosity data (5). Therefore, unlike western countries, countries like India with a vast ethnically different population and higher rate of endogamy, southern part of India, demand extensive study on STR makers with respect to each population and endogamy group (6) limits its applicability vastly. It is likely that the markers may turn uninformative in a population with high consanguinity and reporting will become impossible for such samples and again putting the patients under anxiety.

Alternatively, the recently developed SD-QF-PCR does not require any prior population-based study since it relies on non-polymorphic segmental duplication regions. The segmental duplications are acquired during the course of evolution that is differing in few bases (7). Although their significance is unknown, they are exploited in molecular diagnosis for rapid aneuploidy diagnosis. The diagnosis is also straightforward with no need of any control samples. With automation of end-product analysis through capillary electrophoresis, the method is suitable for high throughput analysis.

Provided these advantages, we aimed to establish this SD-QF-PCR method in our laboratory to complement the routine STR-QF-PCR in prenatal diagnosis.

MATERIALS AND METHODS

Samples and DNA extraction

Control samples ($n = 50$), DS samples ($n = 50$), Trisomy 18 sample ($n = 1$), and Klinefelter syndrome sample ($n = 1$) were studied to optimize the assay. In addition, 100 amniotic fluid samples were also studied along with STR-QF-PCR. DNA extraction was carried out with QIAamp mini DNA kit (Qiagen, Hilden, Germany). Quantification was done on Nanodrop system. Ethical approval was obtained from the institutional ethics committee.

SD-QF-PCR

The primers were obtained from the previously published report (2). For each chromosome, minimum of two regions were selected. Three autosomes—chromosomes 21, 13, and 18—and sex chromosomes were studied. The primer sequence and their product size with respect to the chromosome are given in Table 1. The primers were differentially labeled to facilitate detection on a single run. The labeling was done on the basis of product size.

The multiplex reaction was carried with 10 sets of primer with final concentration as given in Table 2.

TABLE 1. List of Primers Set Used in the Study Along with Their Target Region and Their Amplicon Size

Primer set	Chromosome	Amplicon in bp
Set 1 (FAM)	21	147
	11	128
Set 2 (FAM)	21	259
	6	247
Set 3 (PET)	18	193
	10	161
Set 4 (PET)	18	185
	1	190
Set 5 (NED)	X	215
	Y	221
Set 6 (NED)	X	211
	Y	200
Set 7 (FAM)	13	238
	11	244
Set 8 (FAM)	13	120
	9	102
Set 9 (VIC)	3	315
	X	321
Set 10 (VIC)	18	251
	X	268

TABLE 2. Combination of Primers That Are Used in Multiplex PCR and Their Concentration

Primer	Concentration
Set 1	0.2 μ M
Set 2	0.2 μ M
Set 3	0.4 μ M
Set 4	0.2 μ M
Set 5	0.2 μ M
Set 6	0.4 μ M
Set 7	0.4 μ M
Set 8	0.2 μ M
Set 9	0.2 μ M
Set 10	0.2 μ M

Other than primers, buffer was used at 1X concentration, dNTPs at 0.2 μ M concentration, 2U of Taq polymerase (Merck India Pvt Ltd), 20–50 ng of DNA, and the final volume was made up to 50 μ l. The cycling conditions included initial denaturation at 95°C for 5 min followed by 28 cycles of 95°C for 30 sec, 58°C for 20 sec and 72°C for 40 sec, and a single cycle at 72°C for 5 min.

Fragment analysis

After amplification, the 2 μ l of products was denatured with 8 μ l of Hidi-formamide and 0.5 μ l of Liz500. Denaturation was carried out at the temperature of 95°C for 5 min followed by rapid cooling on ice. Then, loaded on to ABI310 genetic analyzer for capillary electrophoresis, data obtained were analyzed with Gene Mapper software.

Fig. 1. Representative electropherogram obtained after fragment analysis of euploid samples. Normal male (46, XY) sample (A) and normal female (46, XX) sample (B) showing the peaks corresponding to 10 different markers. Double-headed arrow indicates the two different targets amplified from a set of primer. Number above the peak represents chromosome number.

RESULTS

PCR conditions were optimized so that each target chromosome for primer set is amplified with equal efficiency, and the cycles were limited to 28 cycles to avoid further amplification bias at exponential phase. For each chromosome, two different segmental duplication regions were selected and amplified. For each marker, we calculated the allele ratio—ratio of area under peak/height of peak of a primer set—in control and aneuploidy samples.

Autosomal markers

After capillary electrophoresis, area under peak/height ratio was obtained for each set of marker. For control samples ($n = 50$, euploid samples), the ratio was calculated and it ranges between 0.9 and 1.1 (Fig. 1). The mean ratio with standard deviation for each marker is given in Table 3. Similarly, the Down's syndrome ($n = 50$) positive samples were studied and the ratio of 1.4–1.6 was obtained for these samples due to the presence of excess 21 chromosome copy (Fig. 2). The mean ratio is given Table 4. We had

only one case of each trisomy 18 and Klinefelter syndrome. So they were studied in triplicates in order to see the variability. The average value of 1.54 was obtained with respect to trisomy 18, whereas 2.14 were obtained for Klinefelter samples (X/Y markers) (Fig. 2).

Sex chromosome marker

For sex chromosomes, two markers were there to compare X and Y together (set 5, 6) and the two markers for comparison of X with autosomes (set 9 3/X; set 10 18/X). For females, set 5 and 6 resulted in only peaks corresponding to their X chromosome, whereas set 9 and 10 ratio was similar to that of other autosomal markers. For males, set 5 and 6 ratio was similar to that of other autosomes, while the set 9 and 10 markers had a ratio of 1.8–2.1 signifying the presence of single X chromosome.

Applicability in prenatal diagnosis

Hundred amniotic fluid samples were simultaneously subjected to both SD and STR-QF-PCR and we compared the results. All the samples were identified as

Fig. 2. The figure shows the typical results of aneuploidy samples. A trisomy 18 (47, +18) sample (A), A trisomy 21 (47, +21) sample (B), and Klinefelter (47, XXY) sample (C) showing allele ratio of above 1.4 for their respective primer sets signifying the trisomy of the respective chromosome.

TABLE 3. Area Under Peak Ratio for All the Markers with Respect to Control Subjects (*n* = 50)

Markers	Chromosomes	Mean area ratio	Standard deviation
Set 1	11/21	0.94	0.13
Set 2	6/21	0.97	0.11
Set 3	10/18	0.95	0.12
Set 4	18/1	0.97	0.12
Set 5			
Male	X/Y	1	0.08
Female			
Set 6			
Male	Y/X	0.96	0.12
Female			
Set 7	13/11	0.94	0.12
Set 8	9/13	1	0.08
Set 9			
Male	3/X		
Female	3/XX	0.93	0.12
Set 10			
Male	18/X		
Female	18/XX	1.02	0.09

negative for the tested aneuploidy, and the results were comparable between these methods, thus established the applicability of SD-QF-PCR in prenatal setup.

DISCUSSION

This study was aimed to validate the SD-QF-PCR in the diagnosis of aneuploidy. With the help of 50

TABLE 4. Expected Area Under Peak Ratio for Aneuploid Samples

Markers	Chromosomes	Mean area ratio
Set 1	Trisomy 21	21/11 >1.4
Set 2		21/6 >1.4
Set 3	Trisomy 18	18/10 >1.4
Set 4		18/1 >1.4
Set 5	XXY	X/Y >1.8
Set 6	XXY	X/Y >1.8
Set 7	Trisomy 13	13/11 >1.4
Set 8		13/9 >1.4
Set 9	XXY, X0	18/XX 0.9–1.1, >1.8
Set 10		3/XX 0.9–1.1, >1.8

control samples, 50 DS samples, and single trisomy 18 and Klinefelter samples, the methods was validated. For diagnosis, after capillary electrophoresis area under peak was measured for each markers and ratio was obtained for each primer set. The ratio was almost found to be near about 0.9–1.1 for the autosomes. For female sample, the ratio for sex chromosome was a single peak corresponding to X chromosome and X/autosome ratio was similar to that of other autosomal markers (0.9–1.1). In case of male sample, X/Y marker ratio is 0.9–1.1 but autosomal/X ratio was more than 1.8.

The SD-QF-PCR has number of advantages over the existing RAT methods. FISH is not suitable for

high throughput; however, since analysis part is automated, large number of samples can be handled easily. MLPA requires control samples for each batch of samples, the calculation is time consuming, and overnight hybridization makes the procedure bit tedious. QF-PCR, in general, does not require these steps.

However, STR-QF-PCR requires prior knowledge of STR marker informativeness. To make the reporting possible in maximum number of samples, a marker set that are highly informative in the study population should be established (5). Therefore, SD-QF-PCR does not require any such data. It can be used directly in diagnosis after obtaining the primers. Presence of maternal contamination to a minimal extent will not affect results, but excess contamination can lead to misinterpretation or unreliable results.

In our setup, we use our own indigenously developed STR-QF-PCR with markers that have high heterozygosity in our population, though at times the reporting becomes impossible due to uninformative-ness of markers (1). This uninformative-ness is mostly associated with consanguineous marriage, for such cases we used SD-QF-PCR to provide the diagnosis. Unlike STR-QF-PCR, SD-QF-PCR cannot distinguish maternal/paternal origin of non-disjunction as well as maternal contamination. However, STR-QF-PCR cannot diagnose monosomies, whereas SD-QF-PCR can do this work. Thus, both SD and STR-QF-PCR complement each other in many ways.

CONCLUSION

SD-QF-PCR can also be used as a standalone test where STR data are not available for particular group of population for STR-QF-PCR, and also our study on amniotic fluid samples has also proven that SD-QF-PCR is also 100% specific and sensitive for prenatal diagnosis. But precautions should be taken in case

of prenatal samples to avoid maternal contamination through visual inspection.

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AUTHOR'S CONTRIBUTION

Muthuswamy S performed all the laboratory work and drafted the manuscript. Agarwal S designed the study and has given final approval of the article to be published.

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