Development of quantitative-fluorescence polymerase chain reaction for the rapid prenatal diagnosis of common chromosomal aneuploidies in 1,000 samples in Singapore

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ABSTRACT

Introduction: We aimed to develop a rapid quantitative-fluorescence polymerase chain reaction (QF-PCR) to detect common foetal aneuploidies in the Singapore population within 48 hours of sample collection in order to alleviate parental anxiety.

Methods: DNA from 1,000 foetal samples (978 amniotic fluids, 14 chorion villi and eight foetal blood samples) was analysed using a QF-PCR of 19 microsatellite markers located on chromosomes 13, 18, 21, X and Y. A total of 523 samples were archived before the QF-PCR analysis (archived), while QF-PCR was performed and the results obtained within 48 hours of sample collection in the remaining 477 samples (live). The results were confirmed with their respective karyotypes.

Results: In total, 47 autosomal trisomies (T) were found: 30 among the archived (three T13, 12 T18, 15 T21) and 17 among the live (four T18, 13 T21) samples. The QF-PCR results were verified with their respective karyotypes. We achieved 100 percent sensitivity (lower 95 percent confidence interval [CI], 92.8 percent) and specificity (lower 95 percent CI, 99.5 percent), and the time taken from sample collection to the obtaining of results for the 477 live samples was less than 48 hours.

Conclusion: Prenatal diagnostic results of common chromosomal abnormalities can be released within 48 hours of sample collection using QF-PCR. Parental anxiety is alleviated and clinical management is enhanced with this short waiting time.

Keywords: amniocentesis, Down syndrome, FISH, karyotype, short tandem repeat

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INTRODUCTION

Currently, the prenatal diagnosis of chromosomal abnormalities requires conventional cytogenetic analysis that takes 7–14 days before the results are released. This long waiting period typically causes considerable parental anxiety.[1,2] Molecular methods, such as fluorescence *in situ* hybridisation (FISH)[3-8] and quantitative-fluorescence polymerase chain reaction (QF-PCR),[9-14] detect common chromosomal aneuploidies (13, 18, 21, X and Y) that account for 80%–95% of genetic disorders associated with birth defects in live births.[15] These methods release results within 2–3 days of foetal sampling. While FISH is labour-intensive and fluorescence probes are costly, QF-PCR uses the less costly fluorescent-labelled primers for the amplification and detection of polymorphic short tandem repeats (STRs) to determine copy numbers by fluorescence intensities. This technique releases results within 24–48 hours after foetal sampling. This study evaluated the accuracy of QF-PCR as a rapid prenatal diagnostic test in Singapore.

METHODS

Samples of amniotic fluid (AF) (n = 978, 14–24 weeks), chorionic villi (CV) (n = 14, 11–12 weeks) and foetal blood (FB) (n = 8, 22–23 weeks) were collected with informed consent from mothers who underwent prenatal diagnosis in the Antenatal Diagnostic Centre at the National University Hospital, Singapore. The reasons for prenatal diagnosis included advanced maternal age (≥ 35 years), abnormal foetal ultrasonographic results, positive maternal serum test results and family history. The study was approved by the Singapore National Healthcare Group Domain Specific Review Board (D/00/803,

Table I. Details of the 24 STRs used in our QF-PCR study.

Primer mix	Marker	Location	Size	PIC	Het (exp)	Primer sequences (5'→ 3')	Concentration (pmoles)	TA (°C)
3	D13S258	13q21	230-267	0.86	0.876	HEX-ACCTGCCAAATTTTACCAGG(F) GACAGAGAGAGGGAATAAACC(R)	18	65
1	D13S628	13q31–q32	425-470	0.67	0.696	NED-TAACATTCATTGTCCCTTACAGAT(F) GCAAGGCTATCTAACGATAATTCA(R)	20	63
3	D13S631	13q31-q32	192-218	0.73	0.768	6-FAM-GGCAACAAGAGCAAAACTCT(F) TAGCCCTCACCATGATTGG(R)	8	65
1	D13S634	13q14.3–q22	385-440	0.80	0.839	6-FAM-GGCAGATTCAATAGGATAAATAGA(F) GTAACCCCTCAGGTTCTCAAGTCT(R)	10	63
1	D13S742	13q11–q21.1	235-315	0.83	0.847	HEX-ATAACTGGGCTAGGAATGGAAATA(F) GACTTCCCAATTCAGGAGGACT(R)	6	63
2	D18S51	18q22.1	280-310	0.86	0.874	6-FAM–CAAACCCGACTACCAGCAAC(F) GAGCCATGTTCATGACACTG(R)	8	65
1	D18S386	18q22.1–q22.2	330-400	0.85	0.867	HEX-TGAGTCAGGAGAATCACTTGGAAC(F) CTCTTCCATGAAGTAGCTAAGCAG (R)	3	63
1	D18S391	18pter–18p11.22	140-180	0.86	0.876	HEX-GGACTTACCACAGGCAATGTGACT(F) TAGACTTCACTATTCCCATCTGAG (R)	2	63
1	D18S535	18q12.2–q12.3	455-500	0.80	0.804	6-FAM - CAGCAAACTTCATGTGACAAAAGC (F) CAATGGTAACCTACTATTTACGTC(R)	4	63
2	D18S1001	18q11	228-248	0.68	0.723	HEX-AGATATGGGAACAACCTAAGTGTCCATCA(F) CTTCATCTAGTGTAATATCCTCCAGTTCC(R)	6	65
4	D21S11	21q21	225-280	0.79	0.827	6-FAM-TTTCTCAGTCTCCATAAATATGTG(F) GATGTTGTATTAGTCAATGTTCTC(R)	3	55
4	D21S226	21q22.1	440-470	0.48	0.535	HEX-GCAAATTTGTGGATGGGATTAACAG(F) AAGCTAAATGTCTGTAGTTATTCT(R)	4	55
1	D21S1270	21q21–q22.1	285-340	0.86	0.872	6-FAM-CTATCCCACTGTATTATTCAGGGC(F) TGAGTCTCCAGGTTGCAGGTGACA(R)	6	63
4	D21S1411	21q22.3	256-340	0.84	0.869	GTAGATACATACATATGATGAATGC(F) NED-TATTAATGTGTGTCCTTCCAGGC(R)	4	55
3	D21S1412	21q22.2	384-414	0.85	0.868	6-FAM-CGGAGGTTGCAGTGAGTTG(F) GGGAAGGCTATGGAGGAGA(R)	11	65
2	D21S1414	21q11.2-21q21	334-362	0.76	0.793	HEX-AAATTAGTGTCTGGCACCCAGTA(F) CAATTCCCCAAGTGAATTGCCTTC(R)	15	65
1	AMXY	Xp22.1–22.31 Yp11.2	X: 103 Y: 109	NA	NA	6-FAM-CCCTGGGCTCTGTAAAGAATAGTG(F) ATCAGAGCTTAAACTGGGAAGCTG(R)	1.2	63
5	SRY	Y	180	NA	NA	NED-TACAGGCCATGCACAGAGAG(F) TCTTGAGTGTGTGGCTTTCG(R)	6	55
5	XHPRT	Xq26.1	260-302	0.66	0.691	6-FAM-ATG CCA CAG ATA ATA CAC ATC CCC(F) CTC TCC AGA ATA GTT AGA TGT AGG(R)	20	55
5	X22	Xq28/Yq PAR2	194-238	0.80	0.819	6-FAM-TCTGTTTAATGAGAGTTGGAAAGAAA(F) ATTGTTGCTACTTGAGACTTGGTG(R)	20	55
5	DXS6785	Xq	120-220	0.74	0.768	HEX-CGACACAGCAAGTCTCTGT(F) GAGGAGGGTCAGAATCTTG(R)	12	55
4	DXS6789	Xq	100-200	0.73	0.763	6-FAM-TTGGTACTTAATAAACCCTCTTTT(F) CTAGAGGGACAGAACCAATAGG (R)	12	55
5	DXS6803	Xq	110-126	0.59	0.648	NED-GAAATGTGCTTTGACAGGAA(F) CAAAAAGGGACATATGCTACTT(R)	12	55
5	DXS6809	Xq	241-273	0.80	0.825	HEX-TTGGTACTTAATAAACCCTCTTTT(F) CTAGAGGGACAGAACCAATAGG(R)	12	55

F: forward; R: reverse; Het (exp): expected heterozygosity; TA: annealing temperature; HEX: hexachlorocarboxyfluorescein; 6-FAM: 6-carboxyfluorescein; PIC: polymorphism information content; NA: not applicable; STRs: short tandem repeats; QF-PCR: quantitative-fluorescence polymerase chain reaction

19/6/2003) and followed the guidelines of the office of Biomedical Research.

For the cytogenetic analysis, samples of AF 20 ml, CV 5–10 mg and FB 1 ml were cultured for metaphase analysis. 20 metaphase spreads per sample were analysed according to the Association for Clinical Cytogenetics Professional Guidelines.[16] The sensitivity and specificity of QF-PCR for all the samples were compared with their respective karyotypes, which is the "gold standard" in prenatal diagnosis.

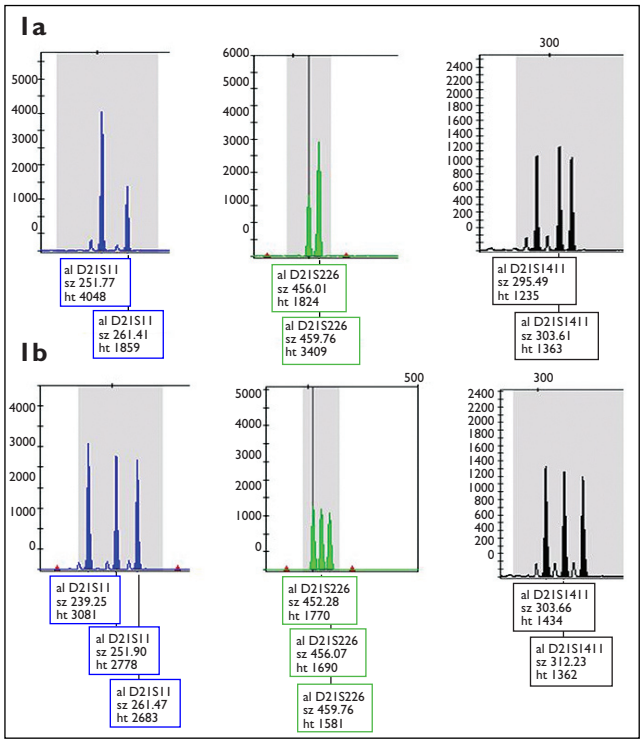

Fig. 1 Fragment sizes in bp are shown on the horizontal axis, while the arbitrary fluorescence units are shown on the vertical axis. Each peak is labelled with marker name, fragment size and height. Electropherogram shows genotyper profiles of trisomy 21 samples. (a) Chromosome 21 markers exhibit two polymorphic alleles in a 2:1 ratio (D21S11) or 1:2 ratio (D21S226) and three polymorphic alleles in a 1:1:1 ratio (D21S1411); (b) Chromosome 21 markers show three polymorphic alleles in a 1:1:1 ratio (D21S11, D21S226 and D21S1411).

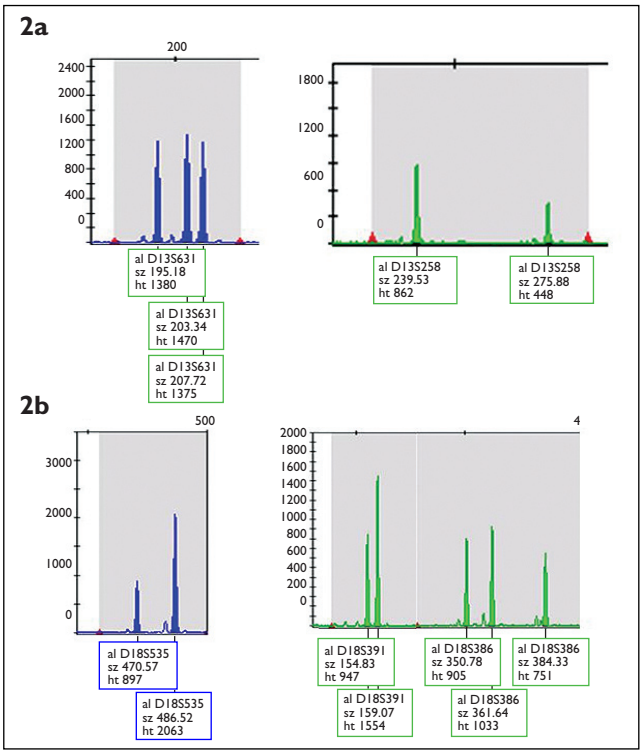

Fig. 2 Fragment sizes in bp are shown on the horizontal axis, while the arbitrary fluorescence units are shown on the vertical axis. Each peak is labelled with marker name, fragment size and height. Electropherogram shows genotyper profiles of trisomy 13 and 18 samples. (a) Trisomy 13 sample with chromosome 13 markers exhibit two polymorphic alleles in a 2:1 ratio (D13S258) and three alleles in a 1:1:1 ratio (D13S631); (b) Trisomy 18 sample with chromosome 18 markers show two polymorphic alleles in a 1:2 ratio (D18S535 and D18S391) and three alleles in a 1:1:1 ratio (D18S386).

Genomic DNA was isolated from 1–2 ml AF, 1–2 mg CV and 200 µl FB samples using the QIAamp DNA blood mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's recommended protocols. All the samples, including bloodstained AF with potential maternal cell contamination (MCC), were washed and resuspended with 200 µl 1XPBS (phosphate-buffered saline, pH 7.4) prior to DNA extraction. A total of 523 DNA samples (518 AF, five CVS) classified as archived, were stored at –80°C for up to four months before QF-PCR analysis. The remaining 477 DNA samples (460 AF, nine CV, eight FB) were classified as live and QF-PCR analysis was performed within 48 hours of sample collection and DNA extraction. QF-PCR analysis was performed without prior knowledge of the foetal karyotypes in this blinded study.

PCR amplification was performed in a total reaction volume of 50 µl containing 25 ng extracted genomic DNA, 0.06–1.8 µmoles of each primer (Proligo-Sigma-Aldrich, St Louis, MO, USA and Applied Biosystems, Foster City, CA, USA) and 1XPCR multiplex master mix (Qiagen GmbH, Hilden, Germany). A total of 19 STR markers located on chromosomes 13 (n = 5), 18 (n = 5), 21 (n = 6), X and Y (n = 3) were amplified in four separate multiplex PCR reactions (labelled as "Primer Mixes (PM)-1, -2, -3 and -4") using fluorescent-labelled and unlabelled primers (Table I). These tetranucleotide STR markers were selected based on their high heterozygosities (Table I). Following initial denaturation at 95ºC for 15 minutes, 28 cycles of denaturation at 94ºC for 30 seconds, annealing for 90 seconds at 63°C for PM-1, 65°C for PM-2 and PM-3, 55°C for PM-4 and extension at 72ºC for 90 seconds for all four PM were performed. This was followed by a final extension step at 72ºC for 10 minutes. Amplification was carried out in a Thermo Hybaid Px2 thermal cycler (ThermoHybaid, Franklin, MA, USA). 2 µl of the amplified allelic fragments were mixed with 9.5 µl formamide and 0.5 µl Genescan-500 Rox (6-carboxy-X-rhodamine) size standards in an optical 96-well reaction plate before denaturation at 95ºC for 2 minutes. This was followed by cooling at 4°C for 2 minutes to prevent re-annealing before capillary electrophoresis with an ABI Prism 3100

Table II. Heterozygosities and PIC in 500 samples.

Chromosome	Marker	Heterozygosity	PIC	No. of alleles
D13	S258	0.876	0.86	17
	S628	0.696	0.67	10
	S631	0.768	0.73	9
	S634	0.839	0.80	11
	S742	0.847	0.83	15
D18	S51	0.874	0.86	17
	S386	0.867	0.85	17
	S391	0.876	0.86	17
	S535	0.804	0.80	12
	S1001	0.723	0.68	8
D21	S11	0.827	0.79	10
	S226	0.535	0.48	6
	S1270	0.872	0.86	13
	S1411	0.869	0.84	17
	S1412	0.868	0.85	14
	S1414	0.793	0.76	9
X/Y	AMXY	NA	NA	NA
	SRY	NA	NA	NA
	X22	0.819	0.80	12
	XHPRT	0.691	0.66	7
	DXS6785	0.768	0.74	10
	DXS6789	0.763	0.73	9
	DXS6803	0.648	0.59	4
	DXS6809	0.825	0.80	9

PIC: polymorphism information content;[17] NA: not applicable

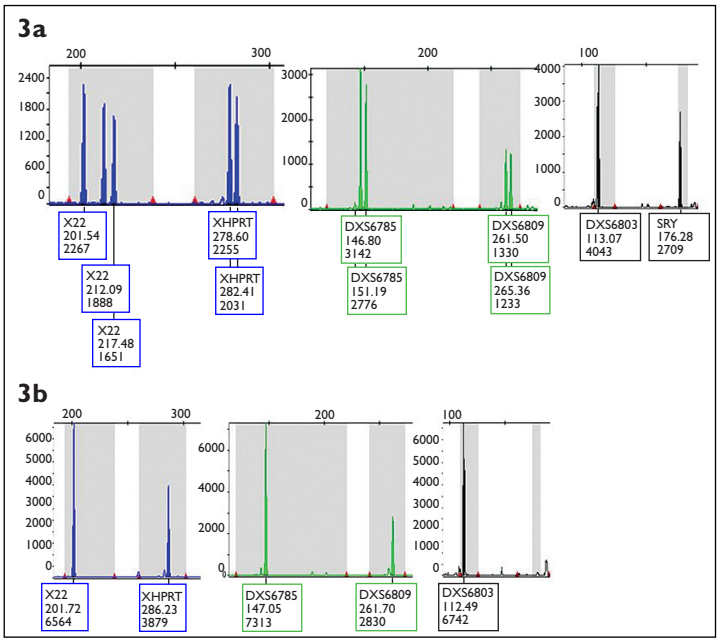

Fig. 3 Fragment sizes in bp are shown on the horizontal axis, while the arbitrary fluorescence units are shown on the vertical axis. Each peak is labelled with marker name, fragment size and height. Electropherogram shows genotyper profiles of sex aneuploidies. (a) Klinefelter syndrome sample shows chromosome X and Y markers with three polymorphic alleles in a 1:1:1 ratio (X22). Polymorphic chromosomal X markers and non-polymorphic chromosomal Y marker (SRY) show normal ratios; (b) Turners syndrome sample shows chromosome X marker with single allele and absence of SRY amplification.

Genetic Analyser (Applied Biosystems, Foster City, CA, USA). GeneMapper version 2.0 (Applied Biosystems, Foster City, CA, USA) was used for data analysis.

RESULTS

Of the 978 AF samples, 913 (93%) were clear fluids, while 60 (6%) were lightly bloodstained and five (0.5%) were heavily bloodstained. Samples were identified as normal when ≥ 2 STRs for each chromosome showed peak height ratios of 0.8–1.4. Chromosomal aneuploidies were identified when ≥ 2 STRs showed peak height ratios of < 0.65 (1:2) or > 1.80 (2:1).[10,17] With this analysis criterion, 47 autosomal trisomies were identified (30 archived with three Patau syndrome, 12 with Edward's syndrome and 15 with Down syndrome) (Figs. 1 & 2). Samples with < 2 STRs that were heterozygous per chromosome were considered "uninformative", and confirmatory tests such as karyotype or FlashFISH, our modified FISH method,[8] were required. Samples with non-specific amplifications and inconsistent dosage ratios across all amplified STRs in all chromosomes were considered to be "inconclusive". For the identification of sex chromosome aneuploidies, six STRs on chromosomes X and Y (Table I) were tested blindly in 100 DNA samples, including 16 sex chromosome aneuploidies (8 with Turner syndrome, 5 with Klinefelter syndrome, 2 XXX, 1 XYY) as positive controls (Fig. 3). All the QF-PCR results correlated with their respective karyotypes, with no false positives or false negatives.

There were 18 uninformative (< 2 heterozygous STRs per chromosome) and eight inconclusive (MCC) results that accounted for a 2.6% failure rate. The polymorphism information content (PIC) and heterozygosities shown in Table II were calculated using the PowerStats version 12.0 freeware (Promega, Madison, WI, USA) in 500 samples. All the STRs showed a high degree of polymorphism (PIC ≥ 0.5) and high heterozygosities.[18]

DISCUSSION

A 100% sensitivity (lower 95% confidence interval [CI] 92.8%) and 100% specificity (lower 95% CI 99.5%) was achieved in the analysis of the 974 informative samples, consisting of AF (n = 955), CV (n = 11) and FB samples (n = 8). 47 autosomal aneuploidies and 16 sex aneuploidies were detected in both the archived (three T13, 12 T18, 15 T21, seven XO, five XXY, two XXX and one XYY) and the live (four T18, 13 T21, one XO) samples. The results of this study support

those of the previous studies in that the rapid detection of common chromosomal aneuploidies using QF-PCR was highly sensitive and specific.[10,13,17,19-21]

Eight samples with MCC were identified as "inconclusive" by inconsistent dosage ratios across all the chromosomes, while 18 samples were concluded as being "uninformative" with < 2 heterozygous STRs per chromosome. In these cases, we waited for a full cytogenetic analysis and subsequently implemented FlashFISH, which allowed us to release the confirmatory results of these samples within the same day.[8]

All results of the live samples were released within 48 hours of sample collection. In total, 19 STRs (six for chromosome 21, five for chromosome 13, five for chromosome 18 and three for the X and Y chromosomes) were used in four separate PCR multiplex reactions. We used a higher number of STR markers than those used in most previously reported studies so as to reduce the number of false positives.[9,10,13,14,17,19,22-27] While false negatives may be falsely reassuring before the release of karyotypes, false positives could have irreversible consequences by potentially leading to the termination of a pregnancy with a healthy foetus. With a 95% sensitivity for QF-PCR in identifying clinically relevant abnormalities and the rapid turnover of results, the targeted diagnosis of at-risk pregnancies with QF-PCR has been strongly recommended in order to minimise the waiting time for genetic counselling and for allaying parental anxiety.[10,17,21,26,28,29]

A common limitation of interphase FISH and QF-PCR is that it cannot detect most structural chromosome abnormalities. However, structural chromosomal abnormalities affecting foetuses are rare and the associated phenotypes can often be detected with ultrasonography. The presence of ultrasonographic markers often calls for a full karotype analysis, where structural chromosomal aberrations can be found.

Despite this limitation, QF-PCR can identify MCC and detect mosaicism of about 20%–30% as compared to interphase FISH and traditional karyotyping. It is also less expensive and allows for a high throughput of samples compared to interphase FISH.[21,24,25,30,31] With its high sensitivity and specificity, as shown in our study, QF-PCR is a reliable and rapid prenatal diagnostic test that is easily affordable for most patients.[21,32]

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