Refining quantitative fluorescent polymerase chain reaction for prenatal detection of X chromosomal anomalies in the major Southeast Asian populations

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ABSTRACT

Introduction: This study aimed to refine the current quantitative fluorescent polymerase chain reaction (QF-PCR) screen to detect X chromosome anomalies for prenatal diagnosis in the major Southeast-Asian populations.

Methods: 100 amniotic fluid samples from Chinese, Malay and Indian origins were subjected to QF-PCR using the X chromosome markers, HPRT, X22 and AMXY, along with the autosomal marker D21S1411.

Results: Out of the 100 samples tested by markers X22 and HPRT, eight samples were homozygous for both markers, of which seven were resolved by comparison with the autosomal marker D21S1411.

Conclusion: 99 percent of samples could be tested for X chromosome copy numbers, increasing the stringency for detection of X chromosome anomalies by QF-PCR. All results were confirmed by cytogenetics.

Keywords: amniotic fluid samples, cytogenetics, prenatal diagnosis, quantitative fluorescent polymerase chain reaction, Turner syndrome, X chromosome abnormalities

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INTRODUCTION

Quantitative fluorescent polymerase chain reaction (QF-PCR) makes use of the fact that short tandem repeats (STRs) are polymorphic at certain loci on all the chromosomes. These chromosome-specific tri-, tetra-, pentanucleotide regions are repeated several times to form DNA sequences of different lengths in the normal population (up to 15–20 alleles). Using fluorescent primers, these STRs are amplified by PCR and the size of the products are analysed.

Fig. 1 Bar chart shows the distribution of the X22 alleles in the Singaporean population.
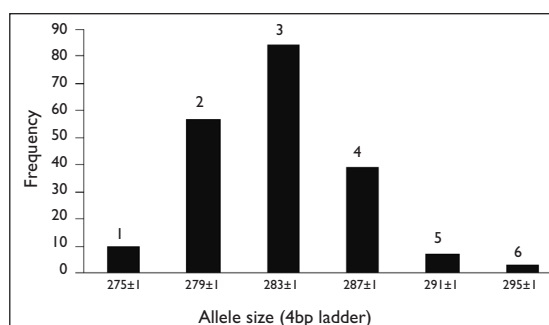

Fig. 2 Bar chart shows the distribution of the XHPRT alleles in the Singaporean population.

For a highly polymorphic marker, more than 80% of a particular population may show heterozygosity, i.e. presence of two differently-sized alleles at that particular locus and these are the ones selected for a screening test. Screens used for QF-PCR for aneuploidy detection in prenatal diagnosis are targeted more for autosomal aneuploidies of chromosomes 13, 18 and 21.[1] QF-PCR for the sex chromosomes X and Y has been used more for determination of gender than as a diagnostic test for aneuploidy detection.[2] This is due to the fact that there are not many polymorphic markers on the X which show a high heterozygosity index, but presently, with the human genome project maps, more markers can be tested out. This enables us to refine the QF-screens to make diagnostic testing for X chromosomal anomalies possible. However, it has to be kept in mind that generally only 2–3 multiplex

Table I. Details of primers used in the QF-PCR sex chromosome multiplex.

Name		Location	Size range (bp)	Sequence 5'–3'	Reference
X22	F	Xq28,Yq12	194–238	HEX-TCT GTT TAA TGA GAG TTG GAA AGA AA	
	R	(PAR2)		ATT GTT GCT ACT TGA GAC TTG GTG	Cirigliano et al[4]
XHPRT	F	Xq26.1	263–299	HEX-ATG CCA CAG ATA ATA CAC ATC CCC	Edwards et al[17]
	R			CTC TCC AGA ATA GTT AGA TGT AGG	
AMXY	F	Xp22.22	106	CCC TGG GCT CTG TAA AGA ATA GTG	Sullivan et al[18]
	R	Yp11.2	112	ATC AGA GCT TAA ACT GGG AAG CTG	
D21S1411	F	21q22.3	256–340	ATGATGAATGCATAGATGGATG	Cirigliano et al[4]
	R			AATGTGTGTCCTTCCAGGC	
				ATA GGT AGA TAC ATA AAT ATG ATG A ³	
				TAT TAA TGT GTG TCC TTC CAG GC	

Table II. 100 amniotic fluid samples tested with the two markers, X22 and XHPRT, in the three different Asian races – Chinese (C), Malay (M) and Indian (I). 8 samples were homozygous for both the markers.

STR marker	X22 (C: M: I)	HPRT (C: M: I)
No. of samples	100 (60: 18: 22)	100 (60: 18: 22)
Heterozygous %	79 (90: 66.6: 72.7)	74 (69.3: 72.2: 72.7)
Homozygous %	21 (10: 33.3: 27.3)	26 (31.7 :27.8: 27.3)
Alleles	12	6

C: Chinese; M: Malay; I: Indian

Table III. Comparison of samples with X22 and XHPRT markers.

Samples	X22	HPRT
59	Heterozygous	Heterozygous
12	Homozygous	Heterozygous
21	Heterozygous	Homozygous
8	Homozygous	Homozygous

Table IV. Analysis of the 8 samples (homozygous with both X22 and XHPRT) compared to the autosomal marker D21S1411.

Sample	HPRT / D21S1412 zygosity	HPRT / D21S1412 ratio
1	Homozygous / Heterozygous	2 to 1:1
2	Homozygous / Homozygous	1 to 1
3	Homozygous / Homozygous	1 to 1
4	Homozygous / Homozygous	1 to 1
5	Homozygous / Heterozygous	2 to 1:1
6	Homozygous / Heterozygous	2 to 1:1
7	Insufficient sample	–
8	Homozygous / Heterozygous	2 to 1:1

reactions with differing markers and PCR run conditions are utilised for rapid, efficient and economic detection of aneuploidies. Hence, we tried out a new marker X22, in addition to the XHPRT and AMXY already present in our earlier screen. Also, by comparing the existing HPRT with an autosomal marker D21S1411, we tried to substantially increase the detection rate of X chromosome copy numbers in our centre without incorporating too many extra STR markers.

METHODS

100 amniotic fluid samples (from 60 Chinese, 18 Malays and 22 Indians), referred mainly for advanced maternal age, were used for this study. A signed form of informed consent was obtained from all the subjects in this study for ethical reasons. DNA was extracted from 2 ml amniotic fluid (QI Amp DNA blood minikit, Qiagen, Valencia, CA, USA) for QF-PCR. The rest of the fluid was used for routine cytogenetic analysis.[3] Fluids with visible blood were not included in this study, with a view to avoiding complications due to possible maternal cell contamination. In the first set of PCR reactions, markers AMXY, X22 and XHPR,[4] were used (Table I). PCR reactions were performed in a final volume of 40 μL containing 5–40 pmol of each primer and 20 μL of Qiagen Multiplex PCR Mastermix and 5 ng of patient DNA template. PCR product amplification and extension were carried out by denaturation at 95°C for 15 mins, 28 cycles at 94°C for 30 s, 55°C for 90 s, 72°C for 90 s followed by 1 cycle of 72°C

for 10 mins. 1-2 μL of PCR product was mixed with 1.9 μL of formamide/ROX mixture, then denatured for 2 mins at 95°C and microsatellite analysis was carried out on the ABI Prism 3100 Avant genetic analyser (Applied Biosystems, Foster City, CA, USA). Electrophoresis results were analysed with the Genemapper version 3.5 software.[5] Reaction 2 using AMXY, XHPRT and D21S1411 was carried out with slightly modified PCR conditions of one cycle of denaturation at 95°C for 15 mins; 27 cycles of amplification 95°C for 30 s, 57°C for 35 s, 72°C for 35 s × 27 cycles followed by 1 cycle of 72°C for 10 mins. The rest of the protocol was similar to reaction 1 and peak height ratios were calculated and compared between the 2 STRs XHPRT and D21S1411.

RESULTS

All samples had a 46,XX karyotype. Of the 100 amniotic fluid samples analysed with the STR markers X22 and XHPRT, 79% were heterozygous for X22. When the data
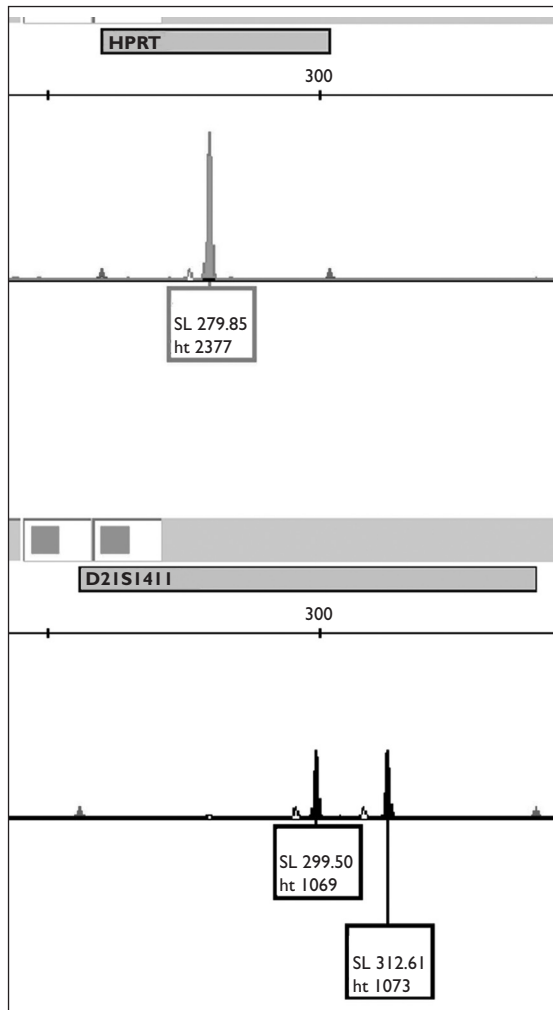
Fig. 3 Comparison of the peak height ratio of HPRT and D21S1411 in a normal female with two alleles homozygous for XHPRT, but heterozygous for the D21S1411 in a ratio 2 to 1:1.
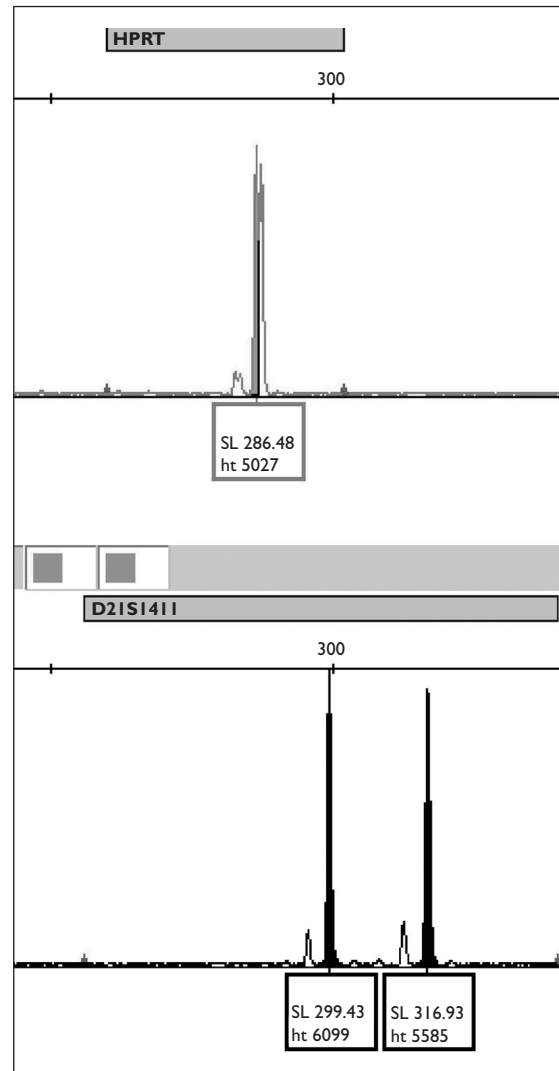

Fig. 4 Comparison of the peak height ratio of HPRT and D21S1411 in a monosomy X (Turner) female with only one allele for XHPRT, but heterozygous for the D21S1411 in a ratio 1 to 1:1.

was sorted according to ethnicity, 90% of the Chinese patients showed heterozygosity for the X22, compared to 66.6% Malays and 72.7% Indians. For the XHPRT marker, the heterozygosity was 74% with the three ethnic groups showing relatively similar values of 69.3%, 72.2%, 72.7% for Chinese, Malays and Indians, respectively (Table II).

There were 12 alleles present for the X22 marker (Fig. 1) and six for the XHPRT marker (Fig. 2). Of the total 100 samples, there were eight samples which were homozygous for both markers and were therefore uninformative for the X copy numbers (Table III). Of these eight samples, seven could be analysed by comparison with the D21S1411 autosomal STR marker on chromosome 21. If a sample was homozygous for XHPRT, but heterozygous for D21S1411, then the expected peak height ratio (PHR) would be 2 to 1:1. Similarly, if a sample was homozygous for both markers, the expected PHR would be 1:1. One

sample did not show any amplification due to an extremely small amount of DNA (Table IV, Figs. 3 & 4).

DISCUSSION

QF-PCR for sex chromosomal anomalies has not been as diagnostic as that for the aneuploidies of chromosomes 13, 18 and 21 due to the paucity of good STR markers along the X chromosome.[6-8] With the sequencing of the human genome, it has now become more possible to try out new markers for all the different regions of the X chromosome. With a view to refining our current QF-PCR screens to enable identification of X chromosomal anomalies, we introduced an additional marker X22 to our already existing set of AMXY, XHPRT and SRY, which were being used mainly for gender identification. Based on our experiments, 92% of the test samples could be analysed by the two markers HPRT and X22, by individual

and joint comparisons. A 79% heterozygosity index for X22 and 74% for HPRT were observed. Within the Asian ethnic groups, the Chinese showed a higher heterozygosity index for the X22 (90%) as compared to the Indians and Malays (~70%). It was interesting to note that a similar study in a Caucasian population showed a heterozygosity index of 87% with the X22 with 12 alleles and 78% with the XHPRT with nine alleles. Hence, STR markers have to be tested for each population before being introduced in a diagnostic multiplex mix as there are major differences in the various ethnic groups.[9,10]

Though introduction of the X22 did improve detection considerably – from 74% to 92%, the eight samples homozygous for both markers remained unresolved. In an earlier study, comparison of the XHPRT with the autosomal marker D21S1411 has been used to detect X copy numbers in a Caucasian population.[11,12] With slight adjustments of the PCR conditions, we successfully managed to compare the XHPRT and D21S1411 and resolve the X copy numbers in seven of the eight samples homozygous for the X22 and XHPRT. The single sample which failed to show amplification was due to an inadequate amount of DNA. Hence, 99% of the samples in this study could be tested for X chromosome copy numbers and were in concordance with the cytogenetic results.

Thus, samples with differing X copy numbers, which form a major group of aneuploidies in prenatal diagnosis, will now be successfully detected by this screen, e.g. 45,X; 47,XXX; 47,XXY; etc.[13,14] However, the drawback in this screen is that structural variants of the Xp arm may not be detected as the X22 and XHPRT are both on the Xq arms. Amelogenin X and Y, on the Xp and Yp terminal regions, is the only STR used in our current screen, but it is a non-polymorphic modified STR marker. This screen will have to incorporate additional markers on the Xp arm, which are now available due to the human genome project information, after testing out the heterozygosity in the Asian population. Low level mosaicism too may go undetected as QF-PCR can only detect mosaicism in proportions greater than 15%.[15,16] However, as Turner syndrome due to monosomy X is one of the major concerns in prenatal detection, this screen would be able to detect that without even the addition of the X22 marker. Hence it has been a very useful study, especially for the Asian population.

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