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Comparison of the Performance of QF-PCR with QPCR as A Rapid Molecular-Based Method for Sex Chromosome Aneuploidies Detection

Authors
Reham Moftah[1], Raymonda Varon[2], Christiane Bommer[3], Véronique Dutrannoy[4] Mohsen Karbasiyan[5], Salah Marzouk[6], Dalal El-Kaffash[7], Heidemarie Neitzel[8]
Affiliation
[1,2,3,4,5,8] Institut für Medizinische Genetik und Humangenetik, Charité-Universitätsmedizin Berlin, Campus Virchow Klinikum, Augustenburger Platz 1, 13353, Berlin, Germany
[1,6,7] Clinical Pathology Department, Faculty of Medicine, Alexandria University, Egypt
[7] Alexandria Regional Center for Woman Health and Development
Corresponding Author
Dr. Reham Moftah
Postal address 1
Charité - Universitätsmedizin
InstitutfürTransfusionsmedizin Research Center forImmunoScience (RCIS, CCM)
Hessische Str. 3-410115 Berlin
Email: *reham-fadl-hassan.moftah@charite.de*
Telephone: 004930450565805
Postal address 2
Clinical Pathology Department, Faculty of Medicine, Alexandria University, El-Khartoom Square, El-Azzarita, Alexandria, Egypt.Telephone: +2 03/4864833

ABSTRACT

Objective: *The high prevalence and variable phenotype of sex chromosome aneuploidies, necessitated the development of a robust method allowing their rapid prenatal diagnosis. Quantitative Fluorescent Polymerase Chain Reaction (QF-PCR) has emerged as a rapid and cost-efficient prenatal diagnostic test for autosomal & sex chromosome aneuploidies. Quantitative real-time PCR (qPCR), an accurate and precise tool for determination of template copy number, represents a potential cost-effective option for sex chromosome copy number detection in laboratories lacking sequencing facilities.*

Methods: *The performance of QF-PCR and qPCR-$\Delta\Delta C_T$ methods for the detection of sex chromosome copy numbers, was evaluated in a retrospective cohort of 56 archival samples; 43 control samples from normal male [n = 19] and female [n = 24] fetuses and 13 sex chromosome aneuploidies. All samples were blindly tested and the results of QF-PCR and qPCR were compared with the original Karyotyping results.*

Results: *qPCR showed 100% sensitivity. Using our QF-PCR sex chromosome primer mix, a case of Turner syndrome was misdiagnosed as normal female. Both methods showed 100% specificity.*

Conclusion: *qPCR is a promising, low cost, rapid tool for sex chromosome copy number detection for further evaluation on a large scale to validate its performance.The introduction of an X/auto some*

paralagous marker and SRY primers to our QF-PCR sex chromosome primer mix will be considered for future studies.

Keywords: *Prenatal, QF-PCR, qPCR, sex-chromosome aneuploidy*

INTRODUCTION

Sex chromosomal aneuploidies are usually diagnosed postnatally in association with specific phenotypic features, associated health problems, diminished fertility, or infertility. Incidence of postnatal detection of sex chromosome aneuploidies is reported to be 1 in 400 live births.[1] Klinefelter syndrome (47,XXY aneuploidy) is the most common disorder of sex chromosomes in humans, with a prevalence of one in 500 male births.[2] Triple X female (47,XXX) accounts for 1:1000 female births.[3]Monosomy X (Turner syndrome), in contrast, has been theorized to be present in 3% of all conceptions, however, 99% of these abnormal fetuses spontaneously abort, usually during the first trimester of the pregnancy, accounting for 7% to 10% of all spontaneous abortions. Approximately 1:2000 to 1:3000 live born girls have Turner syndrome.[4] Other sex chromosomal aneuploidies are much less frequent (48,XXXY, 48,XXYY).[5] The overall incidence of sex chromosome aneuploidies in prenatal settings is 1 in 435, depending on the indication for invasive prenatal testing.[2] However, sex chromosome abnormalities have less severe clinical anomalies than those associated with comparable autosomal imbalances.

Incidental diagnoses of Sex chromosome aneuploidies in routine prenatal invasive testing presents an unexpected finding to the parents. However, early prenatal diagnoses may provide opportunities for early treatment of associated health and developmental problems and represents a chance for better future healthcare of the child aiming at ameliorating the quality-of-life.[6,7]

Identification of X and Y chromosome copy numbers is carried out routinely using conventional cytogenetic analysis which is considered the gold standard. Inter phase Fluorescence In Situ Hybridization [I-FISH] has been established as a rapid prenatal diagnostic test for the most common aneuploidies (chromosomes 13, 18,21, X, and Y); however, I-FISH is both expensive and labor intensive. Quantitative Fluorescent PCR (QF-PCR) has emerged as a rapid and cost-efficient alternative to I-FISH for the prenatal diagnosis of selected chromosome aneuploidies.[8-13] Quantitative real-time PCR has been developed for the detection of deletions/duplications of some genes of the sex chromosomes.[14]Being an accurate and precise tool for determination of template copy number, Quantitative real-time PCR (qPCR) represents a potential cost-effective option for sex chromosome copy number detection in laboratories lacking sequencing facilities.

MATERIALS AND METHODS

Samples

This study was performed at the Institute of Medical and Human Genetics, Charité Universitätsmedizin Berlin, Germany. The

performance of QF-PCR and qPCR-$\Delta\Delta C_T$ methods for the rapid detection of sex chromosome copy numbers, was evaluated in a *retrospective cohort of 56 archival* samples; 43 control samples from normal male [n = 19] and female [n = 24] fetuses and 13 sex chromosome aneuploidies including; Klinefelter syndrome (47,XXY [n = 2]; 48,XXXY [n = 1]), XYY syndrome [n=1], triple X syndrome [n = 3] and Turner syndrome [n = 7]. All samples were blindly tested and the results of QF-PCR and qPCR were compared with the original conventional cytogenetic results. Cell culture, harvesting, karyotyping and DNA extraction were done following standard protocols of the Institute of Medical and Human Genetics, Charité Universitätsmedizin Berlin, Germany.

QF-PCR:

Multiplex PCR using six fluorescently labelled primer pairs (Applied Bio systems) was applied for co-amplification of six markers on chromosome X and Y. The sex chromosome multiplex contained primers for the 3 microsatellite loci that map on the X chromosome; DXS6803, DXS6809 and DXS8377, the X linked hypoxanthine-guanine phosphorribosyltransferase (HPRT) repeat sequence, together with a pentanucleotide repeat, termed X22, which maps in the pseudoautosomal region PAR2 (Xq/Yq) of both the X and Y chromosomes and the modified amelogenin non polymorphic markers present on both X and Y chromosomes (AMXY) (Cirigliano et al. 1999). Data concerning the primers used are shown in table 1. A working primer mix containing all primers at equimolar concentrations (2 µM each primer) was used. PCR was set up in a 25 µL reaction volume containing 12.5 µL 2x Qiagen Multiplex PCR Master Mix (Qiagen), 2 µL working primer mix (160 nM each) and 1 µL template DNA (100 - 200 ng). The PCR cycling conditions for all samples were consistent and performed as previously described(Mann et al., 2004).Amplification was carried out using a Gene Amp® PCR System 9700 thermo cycler (Applied Bio systems). Fragment analysis of the PCR products was carried out using the 3730 DNA Analyzer48 Capillary Array, 36 cm (Applied Bio systems) with Data Collection v2.0 software (Applied Bio systems) and finally the Gene Mapper® Software v3.7 (Applied Bio systems) for fragment sizing and quantification. Each amplified sample (0.5 µL) was added to 9 µL of Ultrapure Hi-Di Formamide (Applied Bio systems) and 0.2 µL of GeneScan-400 Rox size standard (Applied Bio systems) in a Micro Amp® optical 96-well reaction plate (Applied Bio systems). Prior to electrophoresis, the mixture was denatured for 5 min at 95 °C. Finally, samples were loaded into the 3730 DNA Analyzer and subjected to capillary electrophoresis. Normal and trisomic control samples were included in each run.

Peak area measurements were used to calculate allele ratios. Sex chromosome copy number was deduced following the professional guidelines for clinical cytogenetics and clinical molecular genetics, QF-PCR for the diagnosis of aneuploidy best practice guidelines (2012) v3.01.[15]

qPCR-$\Delta\Delta C_T$ Method

Sex chromosome copy numbers detection was done through assessment of dosage ratio of the coagulation factor VIII, procoagulant component (F8) gene, mapped to chromosome X and SRY gene mapped to chromosome Y using qPCR-ΔΔCT method for relative quantification.NHEJ1 gene on chromosome 2 was taken as the endogenous control gene for comparative C_T formula calculation. Primer pairs were designed using the Primer Express® Software v3.0 (Applied Bio systems). Characteristics of the target genes and of the primers used are summarized in Table 2. The target and endogenous control amplification were run in separate tubes. Each 20 µL reaction volume contained 4 µL 5x HOT FIREPol® Eva Green® qPCR Mix Plus (Solis BioDyne), 125 nM of the forward and reverse primer (Invitrogen™) and 10 µL template DNA (50 ng). Each test and normal calibrator sample was tested in triplicate for the target genes and endogenous control gene. The qPCR cycling conditions were set as follows: initial activation at 95°C for 60 s followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 60 s. qPCR was performed in Micro Amp® Optical 96-Well Reaction Plate (Applied Bio systems) on the ABI Prism 7500 Sequence Detection System running the Sequence Detection Software v.1.2.3. (Applied Bio systems). A dissociation curve was run for every plate starting from 60 to 95 °C at a ramp rate of 0.1 °C/s.

Data processing was performed using the SDS software v. 1.2.3 (Applied Bio systems, UK). Delta Normalized reporter (ΔRn) was plotted against cycle number. The threshold was set manually at 0.2 and baseline between cycles 3 - 15. The difference in C_T value between the target and endogenous control genes (ΔC_T value) was calculated for each test and normal calibrator sample. Data were analyzed using the formula: Gene dosage ratio = $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T$ value = ΔC_T Test sample – ΔC_T Calibrator sample

Following Zhu et al. 2009, replicate curves for each sample were checked for uniformity in the amplification plot view either for the target or endogenous control gene, and outliers for which standard deviation (SD) of the C_T value was greater than 0.2 were removed. The ΔC_T value for each sample should be the mean value of at least two replicates; if otherwise, the sample was retested.[16]Prior to adoption of the qPCR-$\Delta\Delta C_T$ method, a validation experiment was performed to ensure equal amplification efficiencies of target genes and the endogenous control gene.

Statistical analysis was performed using IBM SPSS v 19.0. The t-test p-value of < 0.05 was considered statistically significant.

RESULTS

QF-PCR

As shown in Table 3, the QF-PCR results were consistent with cytogenetic results in all 43 normal samples (true negatives) and in 12 out of the 13 sex chromosome aneuploidy samples (true positives); three Klinefelter syndrome cases (Figure 1), a single case of XYY syndrome (Figure 2), three triple X syndrome cases (Figure 3), as well as five out of the six Turner syndrome

cases (Figure 4). The single misdiagnosed sample showed a normal diallelic pattern for DXS6803, whereas all other markers showed a single allele peak (Figure 5) indicating a normal female karyotype; however, this samples was found to have a Turner syndrome karyotype 45,X. The previous behavior reflects a sensitivity of 92.3%. The specificity was 100%.

TABLES

Table 1: Sex chromosome QF-PCR primer multiplex

Marker	Primer Sequence 5´-3´	Size Range (bp)	Repeat size	Cytogenetic location
AMXY	6-FAM-CCCTGGGCTCTGTAAAGAATAGTG (F) ATCAGAGCTTAAACTGGGAAGCTG (R)	X: 106 Y: 112	-	Xp22.22 Yp11.2
X22	6-FAM-TCTGTTTAATGAGAGTTGGAAAGAAA (F) ATTGTTGCTACTTGAGACTTGGTG (R)	194-238	Penta	Xq28 Yq12
XHPRT	6-FAM-ATGCCACAGATAATACACATCCCC (F) CTCTCCAGAATAGTTAGATGTAGG (R)	263-299	Tetra	Xq26.1
DXS6803	HEX-GAAATGTGCTTTGACAGGAA (F) CAAAAAGGGACATATGCTACTT (R)	110-126	Tetra	Xq21.31
DXS6809	HEX-TGAACCTTCCTAGCTCAGGA (F) TCTGGAGAATCCAATTTTGC (R)	241-273	Tetra	Xq21.33
DXS8377	NED-CACTTCATGGCTTACCACAG (F) GACCTTTGGAAAGCTAGTGT (R)	203-246	Tri	Xq28

Table 2: Genes and Primer used for chromosome X and Y copy numbers detections using qPCR

Chromosome	Gene_Exon	Primer sequence 5´-3´	Product size (bp)
Chromosome 2	NHEJ1_Ex6	GGCATGCAGCATTGGTGAT (F) CTTGATGCTTCTGTCCCACTTG (R)	100
Chromosome X	F8_Ex8	GCCAAGAAGCATCCTAAAACTTG (F) GGCGAGGACTAAGGGAGCAT (R)	100
Chromosome Y	SRY	GCCGAAGAATTGCAGTTTGC (F) TGGCTTTCGTACAGTCATCCCT (R)	100

F8: coagulation factor VIII, procoagulant component, SRY: sex determining region Y&NHEJ1*:* non homologous end-joining factor 1 (Ensembl genome browser)

Table 3: Results of testing 56 samples with QF-PCR and qPCR for sex chromosome copy number compared to cytogenetic results

Karyotype	No. by Cytogenetics	No. by QF-PCR	No. by qPCR
46,XX	24	25	24
46,XY	19	19	19
47,XXY	2	2	2
48,XXXY	1	1	1
47,XYY	1	1	1
47,XXX	3	3	3
45,X	6	5	6
Total abnormalities	13	12	13

qPCR- ΔΔCT Method

As shown in Table 3, all 43 control samples normal for X, Y chromosomes copy numbers as well as all 13 Sex chromosome aneuploidy samples tested were correctly confirmed by qPCR-$\Delta\Delta C_T$ method. The sensitivity and specificity were 100%.

Figure [6] shows the mean dosage ratio of chromosome X and Y with the cases grouped according to their karyotype.

The mean chromosome dosage ratio for one copy of chromosome X (n=26) was 1.02 ± 0.15 (range, 0.73 - 1.26) and for 2 copies (n=26) was 2.16 ± 0.31 (range, 1.72 - 2.87) and for 3 copies (n=4) was 3.42 ± 0.31 (range, 3.09 - 3.83). Performing analysis of variance test (ANOVA) test, there was a statistically significant difference between the means of all groups (F = 170.7, P = 0.0001), subsequently, a Tuky post hoc test was performed and it was found that there was a significant difference between group1 (one copy) and group2 (2 copies) ($p<0.001$), group 2 and group 3 (3 copies) ($p<0.001$) and group 1 and group 3 ($p<0.001$).

Y chromosome sequence was negative in 34 samples and positive in 23 samples. The average dosage ratio for one copy (n=22) was 0.92±0.2 (range, 0.72-1.12). A single sample showing two copies of Chromosome Y was tested (46, XYY) and showed the dosage ratio of 1.94.

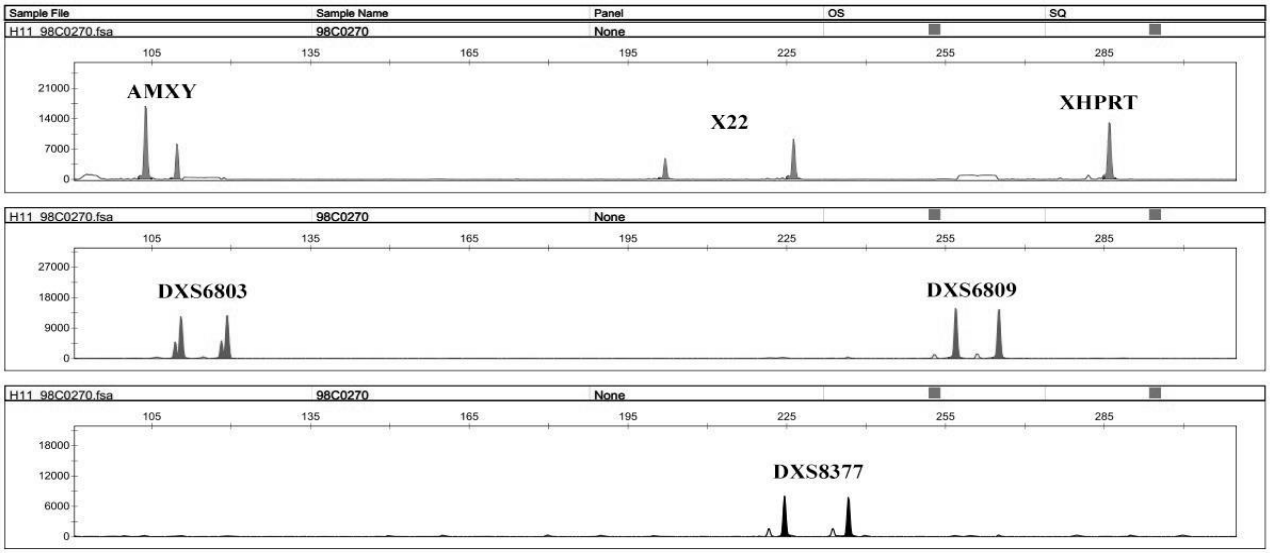

Figure 1 -QF-PCR electrophoretogram of a case of Klinefelter syndrome (47, XXY). AMXY shows 2 fluorescent peaks with the ratio of the X-specific product to the Y-specific product is 2:1. X22 shows two fluorescent peaks with the ratio 1:2. DXS6803, DXS6809 and DXS8377 show normal diallelic pattern. XHPRT is uninformative.

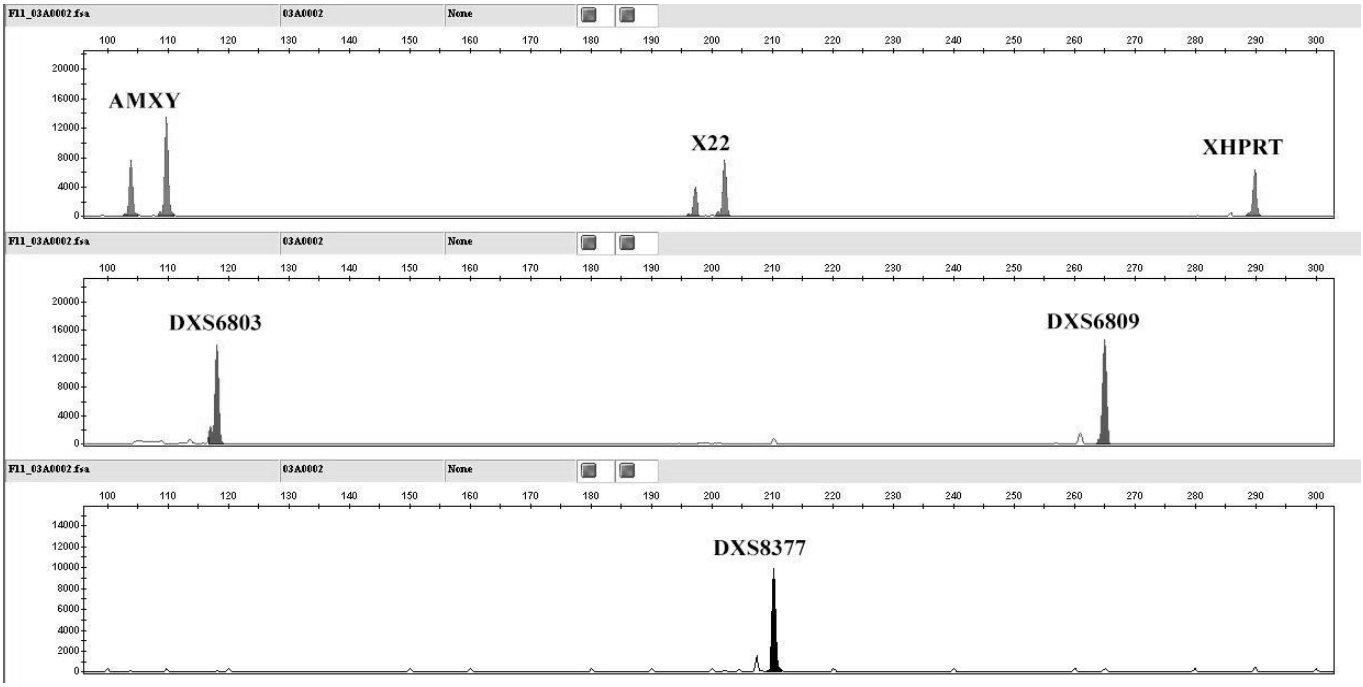

Figure 2: QF-PCR electrophoretogram of a case of XYY syndrome (47,XYY). AMXY shows 2 fluorescent peaks with the ratio of the X-specific product to the Y-specific product is 1:2. X22 shows two fluorescent peaks with the ratio1:2. All X chromosome markers; XPRT, DXS6803, DXS6809 and DXS8377 show single allele peak.

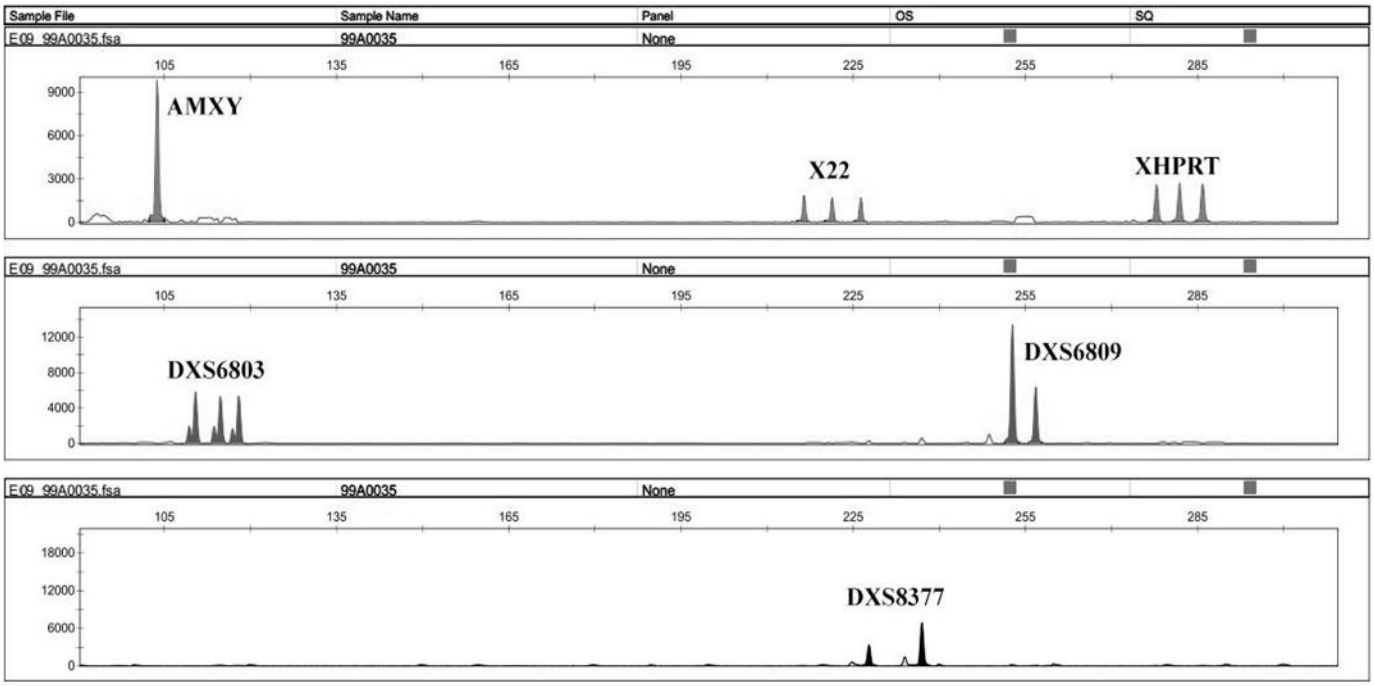

Figure 3: QF-PCR electrophoretogram of a case of Triple X Syndrome (47, XXX) shows triallelic pattern for X22, XHPRT and DXS6803. DXS6809 and DXS8377 show trisomicdialllelic pattern. AMXY shows only the X-specific product.

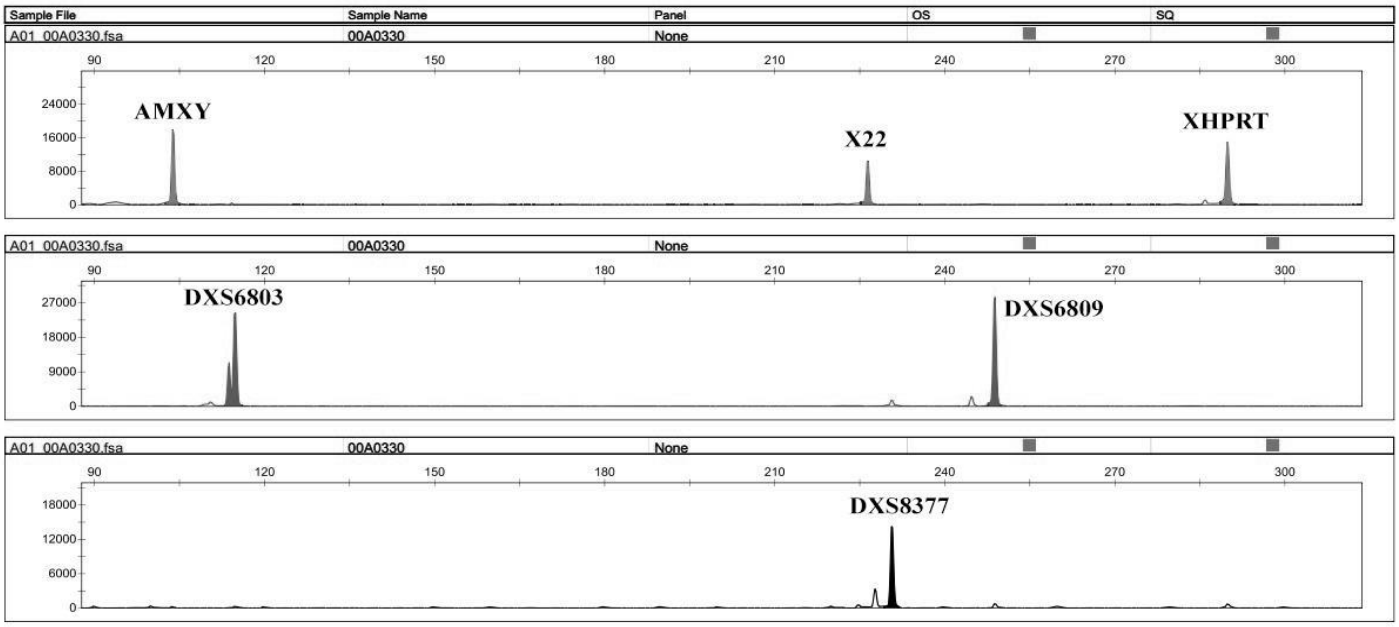

Figure 4: QF-PCR electrophoretogram of a case of Turner Syndrome (45,X). All markers show single allele peak and the AMXY shows only the X-specific product.

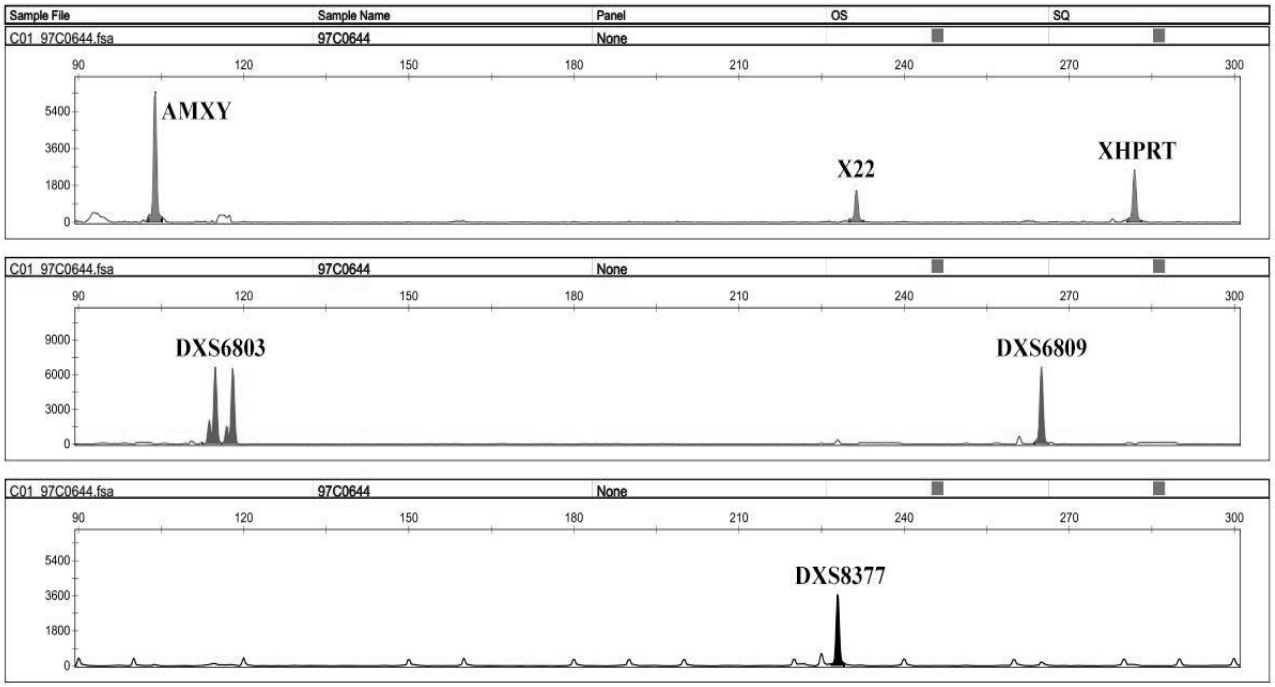

Figure 5: QF-PCR electrophoretogram of a case of Turner Syndrome shows normal diallelic pattern of DXS6803, whereas all other markers show single allele peak.

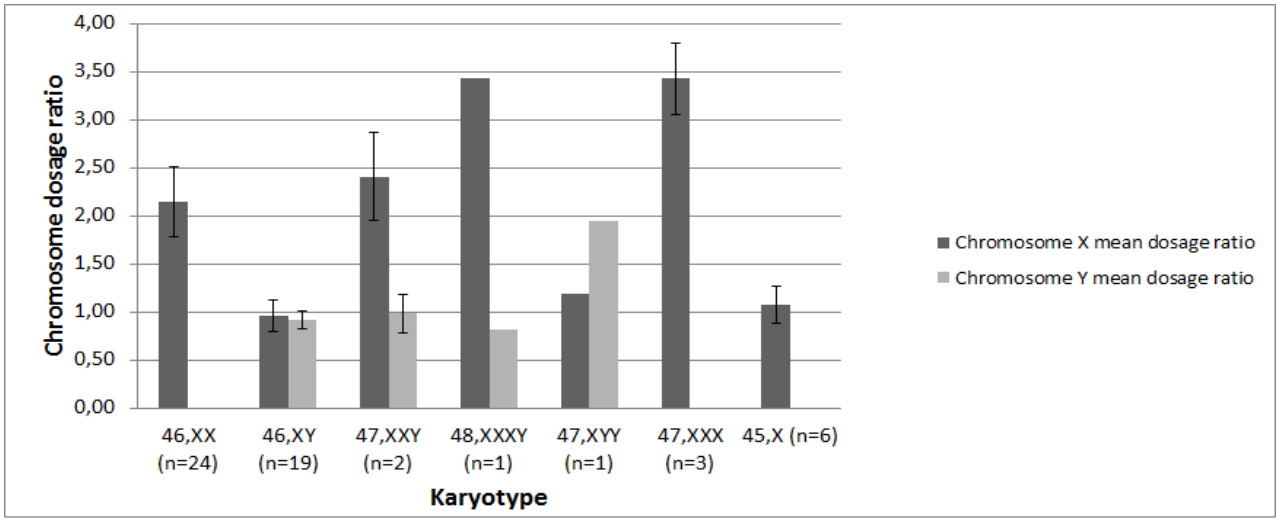

Figure 6: Histogram illustrate the mean dosage ratio ± SD of both chromosomes X and Y by qPCR comparative CT method. Cases are grouped according to their karyotypes.

DISCUSSION

QF-PCR

Our results were in agreement with those reported in the literature.[12, 13, 17-19]

Using QF-PCR, we were able to detect three cases of Klinefelter syndrome and one case of XYY syndrome. The results were confirmed based on the results of two sequences; AMXY and X22,

thus increasing the reliability of the results. Furthermore, according to the professional guidelines for clinical cytogenetics and clinical molecular genetics, QF-PCR for the diagnosis of aneuploidy best practice guidelines (2012) v3.01, it is recommended to confirm a trisomic pattern with at least two markers. The use of X22 allowed this confirmation in Klinefelter syndrome and XYY syndrome samples that otherwise could only be detected using AMXY. However it is strongly recommended to include different Y chromosome sequences, such as SRY, to screen for fetal aneuploidies by QF-PCR in order to increase the reliability of sex detection.[20]

Three Triple X syndrome samples were correctly detected using QF-PCR. In two samples the result was based on trisomic results of the four X chromosome markers as well as pseudoautosomal X22. The third case showed trisomic pattern of the four X chromosome markers, whereas X22 was uninformative. It is worth noting, that the presence of five markers testing for X chromosome copy number in the multiplex assay allowed the proper chromosomal copy number to be deduced confidently in all cases.

The selection of the four X-chromosome markers as well as pseudo-autosomal X22, in this study, was determined based on their high heterozygosity. Therefore, the presence of a single peak for all of these markers, in absence of the Y-specific product of amelogenin, is more likely to result from an X monosomy (Turner syndrome). The high heterozygosity of these markers altogether would markedly decrease the possibility for a normal female to be homozygous for all of them and thus indistinguishable from Turner syndrome.

In this study, five out of six Turner syndrome samples were correctly identified using QF-PCR. The misdiagnosed sample demonstrated a single marker with normal diallelic pattern (DXS6803), while all other markers showed a single fluorescent peak. This QF-PCR pattern was suggestive of a normal female karyotype. However, this was not in agreement with the original karyotype results which confirmed the diagnosis of Turner syndrome. The possible explanation for such discrepancy was a Turner syndrome case with a submicroscopic duplication of DXS6803 or partial chromosomal imbalance. Testing of parental samples using the same marker (DXS6803) for confirmation of submicroscopic duplication was inapplicable.

Sex chromosome assays are now recommended to include an X/auto some paralagous marker, which allows the relative number of X chromosome sequences to be calculated by comparison to auto some sequence copy number. TAF9L (3p24.2/Xq21.1) is now widely used and provides a more confident detection of monosomy X as well as distinguishing between triple X and monosomy X/XX mosaicism.[21] However, our study was conducted before publishing the last version of the QF-PCR for the diagnosis of aneuploidy best practice guidelines (2012) v3.01,

The lack of such marker in our primer mix could be responsible for the misdiagnosed case.

Using QF-PCR, no evidence of Maternal Cell Contamination (MCC) was observed in all tested samples. The characteristic MCC allele pattern

consist of inconclusive diallelic results, or a triallelic result, with a minor third peak with the peak areas of the maternal-specific and fetal-specific alleles equals the area of the shared maternal-fetal allele.[22]

qPCR-ΔΔCT Method

So far, there is little mentioned in literature concerning prenatal detection of sex chromosome aneuploidies using qPCR. However, our results were in agreement with those of Ottesen et al. (2007), who applied the quantitative real-time PCR (qPCR)-based method for Klinefelter syndrome detection. Quantification was done by estimation of the copy number of the androgen receptor (AR) gene mapped to Xq11.2–q12. GAPDH was used as a house-keeping gene for normalization of the AR dosage ratio. This ratio was calibrated to the ratio of a normal male reference DNA. They analyzed samples from 50 individuals, including a healthy male and female controls and patients with Klinefelter syndrome. The reference range for the AR-copy number was established as 0.8–1.2 for one copy and 1.7–2.3 for two copies. The qPCR results were within the reference range in 94% or 97% of the samples with one or two copies of the AR gene, respectively. None of the Klinefelter patients were misdiagnosed as having a karyotype with only one X-chromosome, and in none of the 46, XY males were two copies demonstrated.[23] On the contrary, Ramos et al 2010 used the comparative C_T method for identification of normal male and normal female subjects based on Androgen Receptor (AR) gene copy number. Samples from 31 phenotypically normal men and 26 phenotypically normal women were analyzed. However, he reported a much wider range for chromosome X dosage ratio than ours; being 0.356-1.463 for one copy and 1.484 and 2.809 for two copies.[24]

In our previous report over QF-PCR concerning autosomal aneuploidy detection, QF-PCR showed 100% sensitivity and specificity. Furthermore, it was able to detect MCC and mosaicism when the trisomic cell line was present in adequate concentration.[25] In contrast; qPCR was only able to discriminate trisomic from normal samples with sensitivities of 95.1%, 97% and 100% for trisomy 21, 18 and 13 respectively. The specificity was 100% for all three trisomies. (Unpublished data)

For the sex chromosome copy number detection by qPCR, one, two, three or more folds of each of the sex chromosomes are present relative to that of the normal male calibrator. This makes the detection of sex chromosome copy number by qPCR more reliable than autosomal aneuploidies, which is faced by the problem of the inconclusive 1.5 fold of trisomic relative to the normal disomic chromosome dosage ratio.

However, both methods are at risk of misdiagnosis due to rare occurrence of a deletion or a submicroscopic duplication for one of the tested markers/genes. Therefore, the result of QF-PCR should only be reported as conclusive based on at least two informative markers for either chromosome X or Y to avoid the risk of misdiagnosis. Likewise, testing more than one gene on each chromosome using qPCR is mandatory to guard against misdiagnoses due to partial chromosome imbalances.

Based on our study, we conclude that qPCR is a promising, low cost, rapid tool for sex chromosome copy number detection for further evaluation in a large-scale study in order to validate its performance in terms of accuracy and reproducibility before being introduced for clinical application. However, according to our results, qPCR demonstrated high efficiency and has the potential of being applied as a low cost, rapid prenatal diagnostic test in laboratories not requiring high through put capabilities or those lacking sequencing facilities. The introduction of an X/auto some paralagous marker, TAF9L (3p24.2/Xq21.1) as well as SRY primers to our QF-PCR sex chromosome copy number primer mix would be considered for future studies in order to offer more confident detection of monosomy X and to increase the reliability of sex detection, respectively.

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CONFLICT OF INTEREST: all authors declare no conflict of interest

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