Diagnostic performance of quantitative fluorescence PCR analysis in high-risk pregnancies after combined first-trimester screening

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ABSTRACT

INTRODUCTION: We aimed to determine the diagnostic efficiency of quantitative fluorescence polymerase chain reaction (QF-PCR) in a clinical setting where most of the analyses are performed on chorion villus samples from high-risk pregnancies as determined by combined first-trimester screening.

METHODS: A retrospective study on QF-PCR data from all pregnancies in the Central and North Denmark Regions over a four-year period (n = 2,550) with invasive prenatal testing carried out due to a high risk of carrying a foetus with Down's syndrome. Results of QF-PCR were compared with those obtained by karyotyping. Other supplementary data were obtained from the Danish Foetal Medicine Database and the Danish Cytogenetic Central Register.

RESULTS: QF-PCR for common aneuploidies is fast, has a low failure rate, and is associated with high positive and negative predictive values (PPV, NPV) (> 99.8%) for all analysed abnormal karyotypes except for mosaicism for trisomy 13 (PPV 20%) and sex chromosome mosaic cases (PPV = 40%; NPV = 99.7%)). In 25 (1%) cases, clinically significant chromosome abnormalities other than chromosomes 13, 18, 21, X, and Y were identified by karyotyping.

CONCLUSION: QF-PCR is a rapid and accurate diagnostic method to detect common aneuploidies in high-risk pregnancies. However, the rapid test cannot stand alone as several clinically significant abnormal karyotypes would be overlooked.

FUNDING: not relevant.

TRIAL REGISTRATION: not relevant.

Several studies on large clinical data sets have documented the diagnostic strength of quantitative fluorescence polymerase chain reaction (QF-PCR) for prenatal diagnosis for the common aneuploidies using primarily amniotic fluid (AF) samples [1, 2]. Furthermore, many countries use or have used QF-PCR as a stand-alone test [3, 4]. No studies have thoroughly examined the diagnostic accuracy of QF-PCR in the two-tiered diagnostic strategy currently used in Denmark. In this setting, most of the samples are chorion villus samples obtained from high-risk pregnancies after combined first-trimester screening.

In the Danish national prenatal screening programme, all pregnant women are offered a first-trimester risk assessment for Down's syndrome (DS) in the form of a combined test of pregnancy-associated plasma protein A (PAPP-A) and human chorionic gonadotropin subunit beta (free β-hCG) in maternal blood combined with maternal age and a nuchal translucency (NT) scan [5-7]. An increased risk of carrying a foetus with DS has been defined as a risk assessment in the range > 1:300 [8].

Since 2004, the general policy for invasive prenatal testing of Danish cytogenetic laboratories has been a two-tiered approach, which includes a rapid test like QF-PCR or multiplex ligation-dependent probe amplification (MLPA) for identification of the common trisomies 13, 18, and 21, and sex chromosome aneuploidies followed by karyotyping by standard chromosome analysis. In general, > 80% of invasive prenatal tests in Denmark are performed on chorionic villus samples (CVS) in the first trimester [9].

The objective of this study was to investigate the diagnostic test performance of QF-PCR for detection of trisomy 13, 18, 21, and sex chromosome aneuploidies in high-risk pregnancies ascertained through combined first-trimester screening in order to enable good pre- and post-test counseling to the pregnant women.

METHODS

Participants and data collection

The selected referrals were only those with an increased risk for foetal aneuploidy based on the first-trimester combined test (foetal NT, maternal PAPP-A and free β-hCG levels and maternal age). The threshold for offering invasive testing was 1:300 for trisomy 21 and 1:150 for trisomy 18/13. In a four-year period from October 2008 to September 2011, 2,550 consecutive samples from high-risk pregnancies were received for QF-PCR and standard chromosome analysis from foetal medicine units (national health care) in the Central and North Denmark Regions. A total of 2,177 (85%) CVS and 373 (15%) AF samples were analysed in one laboratory.

Data on maternal characteristics, biochemical markers, ultrasonic markers and outcome of pregnancies

ORIGINAL ARTICLE

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Dan Med J 2014;61(11):A4964

TABLE 1

Characteristics of pregnancies for the total sample set (n = 2,550) and of the three subgroups with aneuploidy of chromosomes 13, 18, 21, sex chromosomes, or other chromosomal abnormality.

	All pregnancies (N = 2,550)	Trisomy 13, 18, or 21[a] (N = 163 ~ 6.4%)	Sex chromosomes aneuploidy[a] (N = 24 ~ 0.94%)	Other abnormal karyotypes[a] (N = 25 ~ 0.98%)
Female foetus, n (%)	1,127 (44)	91 (56)	13 (54)	15 (60)
Male foetus, n (%)	1,423 (56)	72 (44)	11 (46)	10 (40)
Maternal age at sampling, median (range), yrs	33 (16-47)	33^{ns} (18-45)	30^{ns} (21-47)	36* (23-44)
GA at risk assessment, median (range), days	89 (67-148)	89^{ns} (79-102)	88^{ns} (80-99)	86** (79-94)
β-hCG, MoM, median (range)	1.1 (0-6.2)	1.1^{ns} (0.1-5.1)	0.7^{ns} (0.2-3.3)	0.7* (0.1–2.9)
PAPP-A, MoM, median (range)	0.4 (0-4.8)	0.4^{ns} (0.1-3.1)	0.4^{ns} (0.2-2.0)	0.3* (0.1–1.5)
NT, median (range), mm	2.0 (0.7-10.4)	2.0^{ns} (0.9-7.9)	2.7* (1.3-8.2)	1.6* (1.0-4.5)
BMI, median (range), kg/m^2	23 (15-57)	23^{ns} (16-38)	24^{ns} (18-34)	22^{ns} (18-39)
TAT, QF-PCR, median (range), days	1 (1-8)	1^{ns} (1-6)	1^{ns} (1-5)	2^{ns} (1-6)
TAT, karyotyping, median (range), days	14 (1-50)	13* (8-50)	16* (9-26)	21*** (15-41)

BMI = body mass index; GA = gestational age; hCG = human chorionic gonadotropin; MoM = multiple of medians; NT = nuchal translucency; PAPP-A = pregnancy-associated plasma protein A; QF-PCR = quantitative fluorescence polymerase chain reaction; TAT = turnaround time.

*) $p < 0.05$; **) $p < 0.01$; ***) $p < 0.0001$; ns = medians are not significantly different.

a) The p-value of the median test comparing differences of each parameter between each subgroup and the entire group.

were collected from the Danish Foetal Medicine Database and the Danish Cytogenetic Central Register (DCCR). After withdrawal of data, personal identifiers were deleted from the dataset. In Denmark, the use of such de-identified data is exempt from institutional review boards for quality control purposes.

Laboratory methods

Sample preparation

CVS were carefully dissected under a microscope to remove any contaminating maternal decidua. Representative tissue aliquots from different sites of the CVS were collected and pooled for DNA purification. AF samples were inspected for visible blood and, if contaminated, washed with phosphate-buffered saline (PBS) buffer before DNA extraction from 1.5 ml mixed sample. The remaining sample material was used for cell culturing and karyotyping. DNA was extracted from CVS and AF using InstaGene Matrix (BioRad, USA) following the manufacturer's protocol.

Automation yields fast and accurate analytical test results for prenatal samples.

Aneuploidy testing by QF-PCR

All samples were analysed using the Elucigene QST*R (Gen-Probe, UK) assay following the manufacturer's protocol. The assay uses multiplex PCR targeting short-tandem repeat (STR) markers to asses for copy numbers of chromosomes 13, 18, 21, X, and Y. Separation of the PCR fragments was done by capillary electrophoresis (3130XL Life Technologies, USA) and data analysis was done using GeneMarker (Softgenetics, versions 1.95-2.2.0, USA).

Standard karyotyping

Conventional cytogenetic analyses were performed on chromosomes derived from long-term culture of CVS or amniocentesis using standard procedures (Q-banding) with a 450-550 band resolution.

Data analysis

All statistical calculations were done using Microsoft Excel 2003 (Microsoft, Washington, USA). Confidence intervals were calculated according to [10]. In the performed statistical tests, a significance level (alpha) of p-values < 0.05 was considered statistically significant in unpaired, two-tailed Mann-Whitney tests evaluating differences of medians between datasets.

Trial registration: not relevant.

RESULTS

A total of 2,550 prenatal samples were obtained after combined first-trimester screening. Of these, 85% (n = 2,177) were CVS. All samples were processed by QF-PCR and by karyotyping. Risk assessment data including maternal age, gestational age (GA), NT, and maternal plasma multiple-of-median (MoM) values for PAPP-A

TABLE 2

Characteristics of quantitative fluorescence polymerase chain reaction analysis for identifying aneuploidies of chromosomes 13, 18, 21, sex chromosomes, and triploidy compared with standard karyotyping.

Genotype	Test result	Reference method[a], n positive	negative	Sensitivity, %	Specificity, %	PPV, %	NPV, %
Trisomy 13	Positive	10	0	100	100	100	100
	Negative	0	2,540				
Mosaic trisomy 13	Positive	1	4	100	99.8	20	100
	Negative	0	2,545				
Trisomy 18[b]	Positive	29	0	100	100	100	100
	Negative	0	2,521				
Trisomy 21[c]	Positive	117	0	96.7	100	100	99.8
	Negative	4	2,429				
Mosaic trisomy 21	Positive	2	0	100	100	100	100
	Negative	0	2,548				
Triploidy	Positive	8	0	100	100	100	100
	Negative	0	2,542				
XY aneuploidies	Positive	15	0	100	100	100	100
	Negative	0	2,535				
XY mosaic aneuploidies	Positive	4	5	44	99.8	44	99.8
	Negative	5	2,536				

MCC = maternal cell contamination; NPV = negative predictive value; PPV = positive predictive value; QF-PCR = quantitative fluorescence polymerase chain reaction.

a) The reference method was standard chromosome analysis, in a few cases combined with analysis of amniocentesis of the same pregnancy and/or outcome at birth.

b) No cases showing mosaic trisomy 18 were detected, therefore no data are shown.

c) For 2 samples, the QF-PCR results for chromosome 21 were inconclusive and showed trisomy 21 when analysed by standard chromosome analysis. For the other 2 samples, QF-PCR showed normal test results; see main text for further explanations of these.

and free β-HCG were collected from the Danish Foetal Medicine Database. The median (range) values of selected parameters are shown in **Table 1**. In eight (0.3%) cases, QF-PCR failed to give conclusive results due to maternal cell contamination of the samples. In six cases (0.2%), karyotyping failed because of problems with culturing of sample material.

Table 1 shows data from all analysed samples as well as the subgroups of i) abnormal test results involving chromosomes 13, 18, and 21 ii) abnormal test results involving sex chromosomes, and iii) abnormal test results involving other chromosomes. No statistically significant differences were seen between the characteristics of group I and the entire cohort. Significant differences for group II compared with the entire cohort were observed for turnaround times (TAT) for karyotyping. Significant differences for group III compared with the entire cohort were observed for several parameters including maternal age, GA, and increased TATs in group iii. Increased karyotyping between the entire group and subgroups II and III is explained by the increased workload from follow up analysis in this group.

Table 2 shows the standard 2 × 2 tables used to calculate test characteristics for the QF-PCR method for identification of prenatal aneuploidies for chromosomes 13, 18, 21, sex chromosomes and for triploidy. The reference ploidy was based on the reference method karyotyping. In some cases, this was supplemented by analysis of AF of the same pregnancy and/or postnatal outcome. The numbers for true/false positive and true/false negative cases are listed together with the calculated sensitivity, specificity and predictive values for determination of aneuploidy for the each of the indicated karyotypes.

A total of 33 (1.3%) cases had an abnormal karyotype, which would not have been detected by QF-PCR only. After parental testing, eight (0.3%) of these cases were shown to be paternally or maternally inherited chromosome abnormalities associated with a good prognosis (data not shown). In 25 cases (1.0%), abnormal karyotypes with a risk of phenotypic abnormality or with uncertain prognosis were detected. Details on these cases and their outcome are summarised in **Table 3**. The residual risk of an uncommon, clinically significant chromosome aberration if QF-PCR on CVS is normal is thus 25/2,363 ~ 1.1% (95% confidence interval (CI): 0.7-1.6%).

DISCUSSION

Our results show that QF-PCR is a rapid, robust and accurate diagnostic method to detect common aneuploidies in high-risk pregnancies identified at the combined first-trimestre screening. The analysis has a very short TAT and a low rate of test failures. However, a significant proportion (11.8%) of atypical karyotypic abnormalities with likely clinical significance cannot be detected by QF-PCR. Therefore, the fast analysis in the

TABLE 3

Details of prenatal cases with normal quantitative fluorescence polymerase chain reaction results subsequently found to have abnormal karyotypes with risk of phenotypic abnormality or uncertain prognosis (n = 25).

Abnormal karyotype (n)	Identified at follow-up AC (n)	NT, mm (n)	Clinical outcome (n)
Trisomy 4 (1)	No	1.9	IUGR, preterm birth
Trisomy 8 (1)	Nd	1.2	TOP
Trisomy 9 (1)	Yes	1.7	TOP
Trisomy 16 (6)	No (2) Nd (4)	< 3.5 (5) nd (1)	TOP (4) Liveborn (2)
Trisomy 22 (2)	Yes (1) No (1)	2.0 1.2	TOP
Mosaicism for trisomy 2 (2)	No (2)	<3.5 (2)	Liveborn (2)
Mosaicism for trisomy 20 (1)	No	1.7	Liveborn
Deletion 4q (1)	Nd	4.5	TOP
Deletion 7p (1)	Nd	3.4	TOP
Deletion 8p (1)	Nd	4.5	TOP
Deletion 9p (1)	Yes	1.7	TOP
Ring chromosome 15 (1)	Nd	2.1	TOP
Tetrasomi 18p (1)	Yes	nd	TOP
Mosaicism for SMC 22 (1)	Yes	1.3	Liveborn
Mosaicism for SMC (3)	Yes (1) Nd (2)	< 3.5 (3)	Liveborn (3)
Inversion 14q (1)	Nd	1.5	Liveborn

AC = amniocentesis; IUGR = intrauterine growth restriction; Nd = not determined; NT = nuchal translucency; SMC = supernumerary marker chromosomes; TOP = termination of pregnancy.

TABLE 4

After-test risks in different examples of patients with various before-test T13 risk values.

Case no.	Pre-test risk[a], n/N (%)	Post-test risk[b], %
1	1/15,000 (0.007)	4
2	1/150 (0.7)	81
3	1/15 (7)	98

NT = nuchal translucency; T13(mos) = (mosaic) trisomy 13.
a) Before-test risk examples of risk estimates based on NT, maternal blood markers and maternal age.
b) Estimation of post-test risk is calculated as odds(after-test)/(1 − odds(after-test)) = odds before × likelihood ratio/(1 + odds(before) × likelihood ratio). The likelihood ratio is calculated as sensitivity/(1 − specificity) and for T13mos this is (1/1)/(1 − 2545/2549) = 637 based on data in Table 2.

high-risk group is supplemented by full karyotyping. For detection of trisomy 21, the sensitivity and specificity of the QF-PCR analysis were 96.7% and 100%, respectively. No false-positive cases were seen, and the predictive value of a positive test result showing trisomy 21 was 100%. Two cases gave inconclusive QF-PCR test results for chromosome 21 where karyotyping showed trisomy 21. In another two (0.08%) cases, QF-PCR results were false-negatives as trisomy 21 was detected by standard karyotyping.

One of these cases showed partial trisomy 21 (NT 3.5 mm) where a partial duplication of chromosome 21 was translocated to a derivative chromosome 6 (46,XX, der(6). ish add(6)(mFISH+21)). The STR-markers in the QF-PCR analysis were non-informative for the duplicated region of chromosome 21, and hence trisomy 21 was not detected. The other case (NT 2.2 mm) showed trisomy 21 due to an iso-chromosome 21 (46,XX,i(21)(q10).ish iso(21)(wcp21+)). The reason for the false-negative QF-PCR result was thoroughly explored which included analysis for maternal cell contamination of the CVS and sample mix up; no explanation was found. False negative test results have been described in other studies [1, 11].
A plausible explanation is placental mosaicism for a normal cell line. This may also explain why mosaic trisomy 21 in two cases was detected by QF-PCR where standard karyotyping subsequently showed full trisomy 21. Such cases may be avoided if larger fractions of the CVS are used for DNA extraction.

In this study, we found only a limited number of cases with trisomy 13 or 18 (Table 2). This should be kept in mind when evaluating the predictive values for these aneuploidies. Still, for detection of full trisomy 13 (n = 10), both the sensitivity and the specificity of the QF-PCR analysis were 100%, resulting in predictive values of 100%. However, for detection of mosaicism of trisomy 13 (T13mos), we had four cases of false positive test results causing a reduced specificity (99.8%) and low positive and negative predictive values (20% and 80%, respectively). Confined placenta T13mos is a well-known phenomenon [12, 13]. In cases where QF-PCR shows T13mos in DNA extracted from CVS, it is therefore advisable to counsel cautiously and wait for confirmatory analysis although the pregnancy has a high risk based on combined first-trimester risk assessment. From Table 2, the likelihood ratio for T13 in QF-PCR can be calculated to 637. To support the counselling, clinicians can use the knowledge of the before-test risk assessment for T13 for the individual pregnancy together with the calculated positive likelihood ratio for T13mos test results. This is exemplified in **Table 4**. This illustrates that if the before-test risk of T13 was low, the after-test risk remains rather low. On the other hand, if the before-test risk is higher (e.g. 1/15), and QF-PCR shows T13mos, the risk of the foetus being true T13mos increases significantly.

The predictive positive and negative values for detection of non-mosaic sex chromosome aneuploidies were 100%. In general, the sensitivity and specificity of QF-PCR analysis for identifying mosaic sex chromosomes aneuploidies are low. This is a well-known problem for QF-PCR [2] and has been reported for MLPA-based fast analysis as well [14]. The low of sensitivity of QF-PCR makes it unsuitable for reliable detection of sex chromosome mosaicism as demonstrated by the low positive predictive values.

The fast QF-PCR test used in this study is designed to detect the most common prenatal aneuploidies involving chromosomes 13, 18, 21, X and Y. In Denmark, standard chromosomal karyotyping is routinely applied in all cases in order to confirm results from rapid aneuploidy testing and to detect other chromosome abnormalities. In 25 cases (1.0%), clinically relevant abnormal karyotypes not involving chromosomes 13, 18, 21, X, or Y were detected (Table 3). Some of these karyotypes are supposed to be lethal. Caine and co-workers previously reported that abnormal karyotypes with a severe risk of abnormal phenotype were found in 0.25% (AF) to 0.66% (CVS) of the analysed samples [3]. The explanation for the higher detection rate in the present study may be that our cohort only consists of high-risk pregnancies as determined by combined first-trimester risk assessment. The residual risk of having an infant with an uncommon, clinically significant chromosome aberration in a high-risk pregnancy if QF-PCR on CVS for the targeted chromosomes is normal is thus approx. 1%. When only considering the analytical costs, the two-tiered approach is expensive compared with the QF-PCR stand-alone strategy used in e.g. the UK [4]. The Danish national healthcare system continuously funds the two-tiered strategy. An NT > 3.5 mm is considered a significant early-pregnancy finding associated with chromosomal abnormalities [15]. However, in only two of the 25 cases with clinically significant abnormal karyotypes other than chromosomes, 13, 18, 21, X and Y, NT was > 3.5 mm. This finding along with the false-negative cases of trisomy 21 indicate that an increased NT as the sole selection criterion for supplementary analyses in cases where QF-PCR is normal would result in overlooking clinically significant chromosome abnormalities.

CONCLUSION

The two-tiered diagnostic strategy where the rapid QF-PCR analysis is used to detect the common aneuploidies followed by standard karyotyping has again proved its efficiency by showing a high sensitivity and specificity together with positive and negative predictive values > 99.5% for non-mosaic common aneuploidies. The analysis, however, cannot stand alone, as at least 1% of atypical abnormal karyotypes would pass undetected.

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ACCEPTED: 25 September 2014

CONFLICTS OF INTEREST: Disclosure forms provided by the authors are available with the full text of this article

ACKNOWLEDGEMENTS: Jan Frederik Hansen's assistance in extracting and organising our data is warmly acknowledged.

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