ORIGINAL ARTICLE

Clinical performance of non-invasive prenatal testing (NIPT) using targeted cell-free DNA analysis in maternal plasma with microarrays or next generation sequencing (NGS) is consistent across multiple controlled clinical studies

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ABSTRACT

Objective To evaluate the clinical performance of non-invasive prenatal testing for trisomy 21, 18, and 13 using targeted cell-free DNA (cfDNA) analysis.

Methods Targeted cfDNA analysis using DANSR™ and FORTE™ with microarray quantitation was used to evaluate the risk of trisomy 21, 18, and 13 in blinded samples from 799 singleton, twin, natural, and IVF pregnancies. Subjects either had fetal chromosome evaluation by karyotype, FISH, QF-PCR, or karyotype for newborns with suspected aneuploidy at birth. The results of targeted cfDNA analysis were compared to clinical genetic testing outcomes to assess clinical performance.

Results Targeted cfDNA analysis with microarray quantification identified 107/108 trisomy 21 cases (99.1%), 29/30 trisomy 18 cases (96.7%), and 12/12 trisomy 13 cases (100%). The specificity was 100% for all three trisomies. Combining this data with all published clinical performance studies using DANSR/FORTE methodology for greater than 23 000 pregnancies, the sensitivity of targeted cfDNA analysis was calculated to be greater than 99% for trisomy 21, 97% for trisomy 18, and 94% for trisomy 13. Specificity for each trisomy was greater than 99.9%.

Conclusion Targeted cfDNA analysis demonstrates consistently high sensitivity and extremely low false positive rates for common autosomal trisomies in pregnancy across quantitation platforms. © 2015 Ariosa Diagnostics Inc. *Prenatal Diagnosis* published by John Wiley & Sons, Ltd.

Funding sources: This study was supported by Ariosa Diagnostics, Inc.
Conflicts of interest: RS, KW, AB, MB, DH, AS, TM and EW are paid employees of Ariosa Diagnostics, Inc.

INTRODUCTION

Non-invasive prenatal testing (NIPT) using cell-free DNA (cfDNA) from maternal plasma is a highly effective method for prenatal screening for trisomy 21 and other autosomal trisomies.[1,2] The first published proof-of-concept studies analyzed cfDNA using a random whole genome assay with next generation sequencing,[3–5] an approach that was further refined with the use of targeted assays.[2,6–14] The DANSR™ assay enables targeted amplification and analysis of specific regions of clinical relevance, and in combination with the Fetal Fraction Optimized Risk of Trisomy Evaluation (FORTE™) algorithm, forms the key components of the Harmony Prenatal Test™, which has been demonstrated to have high sensitivity and specificity for the assessment of fetal trisomy.[2,6–14] DANSR and FORTE for NIPT have distinct advantages over the random whole genome approach, including lower cost because of more efficient use of sequencing, reduced assay variability, and faster analysis time. Although quantitation of cfDNA for fetal aneuploidy screening to date has mostly relied upon next generation sequencing (NGS), this approach requires costly equipment, reagents, and software and has limited throughput. We recently demonstrated in a comparison of methods study, the equivalency of two DNA quantitation methods, DNA microarrays and NGS, in their ability to accurately quantify the targeted DANSR products for NIPT using the FORTE analysis algorithm.[14] Now, this report directly establishes the clinical performance of targeted cfDNA analysis with DANSR and FORTE using microarray-based quantitation

in a diverse pregnancy population, including singleton and twin gestations as well as pregnancies achieved with *in-vitro* fertilization (IVF) with donor oocyte. We also provide the comprehensive test performance data for the targeted cfDNA DANSR/FORTE assay in screening for the common autosomal trisomies across the two quantitation methods.

METHODS

Study population and clinical evaluation

A total aggregated set of 799 pregnant women subjects were analyzed as part of this cohort study from centers in Sweden, the UK, and the US. All subjects either had diagnostic testing (amniocentesis and/or chorionic villi sampling) and fetal chromosomal evaluation by karyotype, FISH, or QF-PCR or were followed to birth, where evaluation for fetal aneuploidies was performed using newborn examination by a healthcare provider with any suspected aneuploidies at birth confirmed with karyotyping.

Ethics Committee approval was obtained at each participating center, and appropriate informed consent was obtained from all subjects included in this study.

Sample collection

Blood samples from Sweden and the US were collected into either Cell-Free™ DNA BCT tubes (Streck, Omaha, NE) or Ariosa cfD™ tubes (San Jose, CA) and shipped to Ariosa Diagnostics, Inc. (San Jose, CA) within 7 days of collection. Upon receipt at Ariosa Diagnostics, all samples were processed immediately to cell-free plasma and then stored at −20 °C. Samples from the UK were collected into EDTA tubes, processed immediately to cell-free plasma, and stored at −80 °C until shipment to Ariosa for analysis.

Test method

All sample processing from plasma was performed under a blinded protocol in Ariosa Diagnostics' certified and accredited clinical laboratory. A single sample was processed for each subject. As previously described,[6,7,14] cfDNA was purified from each plasma sample, and DANSR products were made from nonpolymorphic assays on each of chromosomes 13, 18, and 21 and polymorphic assays from chromosomes 1–12. The DANSR products from each sample were hybridized to a custom DNA microarray from Affymetrix Inc. (Santa Clara, California), and imaged on an Affymetrix GeneTitan® Multi-Channel instrument.[14] Each patient sample was assayed on a single microarray. For analysis of the subset with matching fetal tissue, pure genomic DNA was isolated from fetal tissue; the DNA was sheared to small fragments with sonication prior to processing through the lab as a cfDNA sample.

Data analysis

FORTE was used to calculate risk scores from the microarray data for trisomy 13, 18, and 21, with the upper and lower risk value capped at 99% and 0.01%, respectively.[14] Samples with risk scores 1% or greater were classified as high risk, and those with risk scores below 1% were classified as low risk.[7,14] Nonpolymorphic DANSR assays on chromosomes 13, 18, and 21 were used to assess relative chromosome representation, while 576 single-nucleotide polymorphic (SNP) DANSR assays were used to estimate fetal fraction.[7,14] Fetal fraction calculation was described previously.[7] Briefly, SNPs were optimized for minor allele frequency in the MapMap-3 dataset (http://hapmap.ncbi.nlm.nih.gov/). Informative polymorphic loci were defined as loci where fetal alleles differ from maternal alleles. Because DANSR exhibits high allelic specificity, informative loci were readily identified when the fetal allele proportion of a locus was measured to be between 1 and 25%. All informative loci for each sample were used to compute the fetal fraction estimate. For the determination of trisomic risk of pure fetal genomic DNA processed with the DANSR methodology, the summarized median $\log_2$ ratio for each of chromosome 13, 18, and 21 signal was computed for each sample. An expected disomic sample would have a median $\log_2$ ratio of 0, while a trisomic sample would have median $\log_2$ ratio of higher than 0 in proportion to the fetal minor source fraction.

RESULTS

Patient characteristics

A total of 799 subjects were included in the study, comprising 759 singleton and 40 twin pregnancies, with 5 *in vitro* fertilization pregnancies, 4 of which were non-self egg donors. As shown in Table 1, the cohort included 152 confirmed cases of fetal aneuploidies. The median maternal age of the study cohort was 36 years (IQR 32–40), and the median gestational age at the time of the blood draw was 16 weeks (IQR 13–19). The median fetal fraction of the cohort was 13.8% (IQR 10.7 – 16.9).

Test performance using DANSR and FORTE with microarray quantitation

A risk score of trisomy 21 (T21), trisomy 18 (T18) and trisomy 13 (T13) was obtained for 791 out of 799 subjects, as 8 of the subjects did not produce a risk score because of insufficient fetal cfDNA. As shown in Table 2, all 641 euploid pregnancies which produced a risk score were classified correctly as low risk for all three trisomies (specificity 100%, 95% CI: 99.4 – 100%). For the trisomy 21 cases, 107 out of 108 were classified correctly as high risk for trisomy 21, with one trisomy 21 case classified as low risk (sensitivity 99.1%, 95% CI: 94.9 – 99.9%).

Table 1 Characteristics of study subjects

Characteristic	Values
Euploid subjects	647
T21 subjects	108
T18 subjects	32
T13 subjects	12
Twin pregnancies (IVF)	40 (1)
Singleton pregnancies (IVF)	759 (4)
Maternal age, year, median (IQR)	36 (32–40)
Gestational age, weeks, median (IQR)	16 (13–19)
Fetal fraction, %, median (IQR)	13.8 (10.7 – 16.9)

Table 2 Test Performance of DANSR/FORTE using microarray quantitation

Diagnostic outcome	Subjects	Test high risk (chr)	Test low risk (chr)
Total subjects with results	791		
Euploid subjects	641	0/0/0 (21/18/13)	641/641/641 (21/18/13)
T21 subjects	108	107 (21)	1 (21)
T18 subjects	30	29 (18)	1[a] (18)
T13 subjects	12	12 (13)	0 (13)

[a]Euploid status of this sample is supported by DANSR/FORTE analysis of pure fetal genomic DNA.

Table 3 Comprehensive clinical performance of DANSR/FORTE

Characteristic	T21 test values	T18 test values	T13 test values
Total subjects	23 155	22 399	14 243
True positives	418	147	30
False positives	10	5	3
True negatives	22 724	22 243	14 208
False negatives	3	4	2
Sensitivity (95% CI)	99.3 (97.9–99.8)	97.4 (93.4–99.0)	93.8 (79.9–98.3)
Specificity (95% CI)	99.96 (99.92–99.98)	99.98 (99.95–99.99)	99.98 (99.94–99.99)

Of the 30 trisomy 18 cases which produced a risk score, 29 were classified correctly as high risk for trisomy 18, with a single trisomy 18 case classified as low risk (sensitivity 96.7%, 95% CI: 83.3 – 99.8%). All 12 trisomy 13 cases were correctly classified as high risk for trisomy 13 (sensitivity 100%, 95% CI: 75.8 – 100%).

For the single trisomy 18 case that was classified as low risk, a fetal tissue sample was available and used for testing with the DANSR assays and microarray. The pure fetal genomic DNA sample, which was confirmed based on quantification of Y chromosome from the male fetus, produced a median log ratio of zero for the ratio of chromosome 18 signal to the other chromosomes, indicating that the fetal tissue sample did not have trisomy 18. Accounting for this, there were only 29 trisomy 18 cases, all of which were correctly identified.

Comprehensive DANSR/FORTE clinical test performance

Combining this cohort dataset with data from nine previously published clinical performance cohort studies using the DANSR/FORTE method provides the test performance in over 23 000 pregnancies,[2,7–14] including 421 trisomy 21, 151 trisomy 18, and 32 trisomy 13 cases. As shown in Table 3, the demonstrated sensitivity for trisomy 21 is 99.3% (95% CI: 97.9 – 99.8%), while for trisomy 18 and trisomy 13, the sensitivities are 97.4% (95% CI: 93.4 – 99.0%) and 93.8% (95% CI: 79.9 – 98.3%). The clinical specificity for all three trisomies are greater than 99.9%, with the 95% confidence interval lower limit of 99.9%.

DISCUSSION

This study establishes high sensitivity and specificity of a targeted cfDNA analysis using DANSR and FORTE with microarray-based quantification in a clinically representative pregnancy cohort. In this cohort, we observed sensitivities of 99.1% for trisomy 21, 96.7% for trisomy 18, and 100% for trisomy 13. All euploid pregnancies were correctly identified as low risk (100% specificity).

This blinded study had complete outcome information (either by invasive testing or by newborn exam) for each sample and included overall a large number of aneuploid cases (108 trisomy 21, 30 trisomy 18, and 12 trisomy 13). Consistent with previously reported clinical validation studies, pregnancies with known fetal mosaicism were not included. In contrast to many previous studies, this cohort was intentionally diverse and included singleton and twin pregnancies as well as IVF pregnancies with self and donor ovum.

Two cases of fetal trisomy (one trisomy 21 and one trisomy 18) yielded low risk results. Discordance between cfDNA analysis and fetal genotype has been reported previously with case reports suggesting potential biological mechanisms, such as confined placental mosaicism.[15,16] The discordant trisomy 18 result reported here was investigated further in our laboratory as we had access to pure fetal genomic DNA for this pregnancy. In this case, a low-risk cfDNA result was reported, but clinical records reported a male fetus with trisomy 18. A modified FORTE analysis of the pure fetal genomic DNA was consistent with a male fetus with no evidence for trisomy 18, supporting the original low risk result obtained from the cfDNA. This suggests that the reason for the discordance is likely incorrect clinical annotation of the case, rather than the tissue type represented by the cfDNA.

Several well-controlled studies have been published on the performance of the Harmony Prenatal test, all using the targeted DANSR assay in combination with the FORTE analysis algorithm. These results are entirely consistent with previously reported data for targeted cfDNA analysis using DANSR

and FORTE with next generation sequencing quantification.[2,7–14] By combining this dataset with all data from previously published studies assessing test performance, a broad picture of the clinical performance for targeted cfDNA analysis using DANSR and FORTE is provided in over 23 000 pregnancies,[2,7–14] including high-risk and low-risk pregnancies, singletons and twins, and IVF pregnancies.

Overall, demonstrated sensitivity for trisomy 21 is greater than 99%. For trisomy 18 and trisomy 13, the sensitivities are 97.4% and 93.8%, respectively, albeit with wider confidence intervals given the fewer number of cases because of lower prevalence of these conditions. The sensitivity is in line with a previously reported meta-analysis of all published clinical studies for all NIPT methods.[1] The specificity for each of trisomy 21, 18, and 13 is greater than 99.9% and has a tight confidence interval given the large number of non-trisomy cases evaluated across the studies. The high specificity and in turn low false positive rate provide for a high positive predictive value. It should be noted that because of the low prevalence of fetal trisomy conditions, the negative predictive value of testing for each trisomy exceeds 99%.

The included studies are either cohort studies with known pregnancy outcome or blinded prospective studies with complete follow-up. Registry reports or other studies with incomplete follow-up have not been included. Registries usually lack complete follow-up data and therefore can introduce bias into any analysis which tries to estimate test performance from such studies.

The introduction of NIPT has led to fairly quick adoption in clinical practice. This study provides further support on the high accuracy of NIPT as compared to conventional screening methods using serum markers and/or ultrasound markers. The primary barrier to widespread adoption of NIPT for the general pregnancy population appears primarily one of cost and reimbursement as the clinical utility is readily apparent.

ACKNOWLEDGEMENTS

Perinatal Laboratory Hospital (Maria Hallingstrom, Malin Karlsson) at Sahlgrenska University Hospital, Barnmorskegruppen AB, Norra Alvsborg County Hospital, Sodra Alvsborg County Hospital. Ariosa Diagnostics (Coleen Hacker, Naiping Shen).

WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- NIPT using targeted cfDNA analysis with NGS has high sensitivity and specificity for fetal trisomy 21 and other autosomal trisomies.

WHAT DOES THIS STUDY ADD?

- This study establishes the high sensitivity and specificity of NIPT using targeted cfDNA analysis with a microarray quantitation platform, and demonstrates that clinical performance is based on the targeted cfDNA analysis method rather than the quantitation method, as the performance using microarray is comparable with performance from previous NGS studies.

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