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First trimester Down syndrome screening with dried blood spots using a dual analyte free beta hCG and PAPP-A immunofluorometric assay

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Objective To determine the effectiveness of first trimester Down syndrome screening with dried blood spots using a dual analyte free beta human chorionic gonadotrophin (hCG)/pregnancy-associated plasma protein A (PAPP-A) immunofluorometric assay.

Method An initial retrospective study of 54 Down syndrome cases and 1064 control specimens was performed followed by a series of 146 513 specimens from routine screening. Detection rates at a fixed 5% false-positive rate were determined separately based on reference data from the retrospective study set and then adjusted based on the routine screening study set.

Results On the basis of the retrospective analysis, the estimated detection rate using free beta hCG, PAPP-A and maternal age varied from 78% at 9 weeks of pregnancy to 70% at 13 weeks of pregnancy. Using a combined protocol, including NT, the detection rate varied from 92 to 90% between 9 and 13 weeks of gestation. Adjusting distribution parameters based on the routine screening dataset reduced the detection rate by at most 1%.

Conclusion Analysis of free beta hCG and PAPP-A using a dual analyte dried blood spot assay is an effective tool in Down syndrome screening, adding an important option for those considering implementation or modification of existing prenatal screening programs. Copyright © 2011 John Wiley & Sons, Ltd.

KEY WORDS: free beta hCG; PAPP-A; maternal serum screening; dried blood; fetal ultrasound

INTRODUCTION

Although Down syndrome screening has historically been conducted during the second trimester of pregnancy, screening in the first trimester using free beta human chorionic gonadotrophin (hCG), pregnancy-associated plasma protein A (PAPP-A) and nuchal translucency (NT) has become clinically accepted (Krantz *et al*., 2000; Malone *et al*., 2005; Nicolaides *et al*., 2005; Perni *et al*., 2006; Kirkegaard *et al*., 2008; Schaelike *et al*., 2009). Initial controversy over whether to use total or free beta hCG in first trimester screening has now been resolved with the overwhelming majority of centers using free beta hCG. Over 20 large scale population-based studies of the free beta hCG, PAPP-A and NT protocol have been published demonstrating detection rates of approximately 90% for a 5% false-positive rate (Krantz *et al*., 2000; Malone *et al*., 2005; Nicolaides *et al*., 2005; Perni *et al*., 2006; Kirkegaard *et al*., 2008; Schaelike *et al*., 2009).

The combination of biochemistry and NT has been shown to perform more effectively at earlier gestational ages (Spencer *et al*., 2003; Borrell *et al*., 2004; Evans *et al*., 2007; Kirkegaard *et al*., 2008). As a result, protocols have been developed in which blood is drawn during 9–11 weeks of pregnancy followed by NT assessment during 11–13 weeks of pregnancy. A significant benefit to this protocol, aside from the improved performance resulting in higher detection rates and lower false-positive rates, is that the patient can receive an immediate result at the conclusion of the ultrasound examination because the biochemical testing will have already been completed. The patient can thus choose to undergo chorionic villus sampling (CVS) at this earlier date rather than waiting for an amniocentesis. Not surprisingly, a recent study by Fox *et al*. (2010) has shown that patient satisfaction is improved in situations where they can make decisions at earlier time points in the pregnancy.

The use of dried blood spot technology in conjunction with the first trimester protocol is a major enabler in adopting this early screening protocol. Dried blood spot (DBS) collection can be facilitated in centers where phlebotomists may not be available; and the patient can draw the blood themselves by a simple finger stick method. No less important than the collection method is an improved screening performance because the drying process stabilizes the protein, effectively decreasing the population standard deviation (SD) (Spencer *et al*., 1993).

We have successfully utilized dried blood spot free beta hCG/PAPP-A and free beta hCG/alpha fetoprotein

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assays in first and second trimester screening, for over 15 years. Recently, we adapted the serum-based free beta hCG/PAPP-A dual analyte immunofluorometric DELFIA assays for use with dried blood spot protocol. In this study, we demonstrate the performance and effectiveness of the assay.

METHODS

A retrospective analysis was performed on 54 known Down syndrome cases and 1064 unaffected controls from specimens submitted to our laboratory and stored at −20 °C for up to 3 years. The Down syndrome cases were from a single institution in which abnormal karyotypes were identified by reviewing all cytogenetic studies performed following CVS and amniocentesis, on tissue following miscarriage or abortion, and in the neonatal period and were available in our freezer at the time of the study (Chasen and Krantz, 2011). The average gestational age was 81.1 days (SD: 5.47) and the average maternal age was 33.5 years (SD: 4.51) in the unaffected group. The population was 62.5% Caucasian, 13.2% Asian, 9.4% Hispanic, 4.6% African American and 10.3% other ethnic groups. In the Down syndrome group, the average gestational age was 82.1 days (SD: 5.49) and the average maternal age was 37.5 years (SD: 3.52). Among the Down syndrome group, 77.8% were Caucasian, 11.1% Asian, 5.6% Hispanic, 1.9% African American and 3.7% other ethnic groups. After the conclusion of the retrospective evaluation, routine screening began.

After 6 months, a series of 146 513 specimens analyzed in a clinical setting were evaluated to assess whether the distribution of free beta hCG and PAPP-A observed was similar to the retrospective analysis. The average gestational age in this group was 86.0 days (SD: 6.21) while the average maternal age was 31.5 years (SD: 5.71). In this group, 63.6% were Caucasian, 5.9% Asian, 13.0% Hispanic, 11.7% African American and 5.8% other.

Samples were analyzed with the AutoDELFIA dual analyte free beta HCG/PAPP-A assay, which is a solid phase, direct punch and 'sandwich-type' immunoassay. The assay utilizes monoclonal antibodies immobilized on microplates and directed against PAPP-A and free beta hCG. During the elution process, PAPP-A and free beta hCG bind to the coated wells and interact with europium and samarium labeled monoclonal antibodies to PAPP-A and free beta hCG, respectively. The addition of enhancement solution causes dissociation of the Eu or Sm from the antibody where they form highly fluorescent chelates with components of the enhancement solution. Fluorescence is proportional to the concentration of PAPP-A and free beta in the sample. Calibrators and controls, standardized against the First International Reference Preparations 75/551 (free beta hCG) and traceable to the formerly extant PAPP-A IRP 78/610, are present on all assays. The inter-day, inter-assay and intra-assay coefficient of variance are presented in Table 1.

Table 1—Assay variance summary for low and high concentration specimens

	Free beta hCG		PAPP-A	
	Low (%)	High (%)	Low (%)	High (%)
Intra-assay	8.6	6.2	7.5	7.1
Inter-assay	3.1	4	6.3	7.6
Inter-day	3.6	4.8	1.8	4.8

hCG, human chorionic gonadotrophin; PAPP-A, pregnancy-associated plasma protein A.

For the retrospective analysis, gestational age-specific medians were determined using the subgroup of Caucasian patients and multiples of the median (MoM) values were determined. MoMs were then adjusted for weight and ethnicity using the laboratory's preexisting adjustment equations.

Parameters for log-Gaussian distributions were calculated for the retrospective samples as follows. The Down syndrome mean MoM for each analyte was determined using the log of the regressed gestation-specific median MoM value. The unaffected mean was set equal to 0 for both analytes. SD was determined by subtracting the 10th percentile from the 90th percentile on a log scale, and dividing by 2.563. For the Down syndrome sample distribution, the SD was adjusted to account for the variation of median MoM with gestation by determining the SD for each week of gestation and then taking the average variance weighted by the number tested in the week. Correlation coefficients between free beta hCG and PAPP-A were determined after excluding specimens beyond three SDs from the mean. For the distribution parameters for NT, published data was used (Cuckle and Benn, 2010) and the correlation between each biochemical marker and NT was set equal to 0.

For the routine samples, the unaffected parameters were determined in the same manner as described for the retrospective dataset. For the Down syndrome distribution the means were set equal to those determined in the retrospective dataset while the SD for each analyte and correlation of free beta hCG and PAPP-A were adjusted based on differences in the unaffected parameters determined in the routine sample set compared to the retrospective series. Specifically, the variance for each analyte was determined by adding the difference between the unaffected variance in the routine screening dataset and the retrospective dataset and adding that difference to the Down syndrome variance in the retrospective dataset. The SD was then determined by taking the square root of the variance. The correlation between free beta hCG and PAPP-A was determined by adding the difference in the unaffected covariance between the routine screening and retrospective datasets to the Down syndrome covariance and then dividing by the associated SD values. For NT, the same published parameters described for the retrospective dataset were used (Cuckle and Benn, 2010).

False positive and detection rates were determined by numerical integration of the Gaussian distributions and the age distribution of live births in the USA in 2004.

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Table 2—Observed and regressed medians for free beta hCG and PAPP-A in Caucasian patients

			Free beta hCG (ng/mL)		PAPP-A (mIU/mL)	
Gawk	*N*	Median days	Observed	Regressed	Observed	Regressed
9	12	67.5	174.27	112.43	0.545	0.459
10	132	73	92.44	96.26	0.645	0.647
11	293	81	76.14	78.34	1.03	1.066
12	210	85	74.15	71.2	1.43	1.368
13	18	93	59.37	59.57	2.125	2.254

GA, gestational age; hCG, human chorionic gonadotrophin; PAPP-A, pregnancy-associated plasma protein A.
Regression formulas: FB-hCG $= 10^{(4.002-1.982\times\log 10\ (\text{GA in weeks}))}$, PAMoM $= 10^{(-2.1673+0.1897\times(\text{GA in weeks}))}$.

Table 3—Observed and regressed free beta hCG and PAPP-A MoM values in Down syndrome pregnancies

			Free beta hCG			PAPP-A		
Gawk	N	Median days	Observed	Regressed[a]	Meta-analysis[b]	Observed	Regressed[a]	Meta-analysis[b]
9	0	—	—	1.38	1.67	—	0.36	0.38
10	10	73.5	1.75	1.48	1.77	0.48	0.4	0.43
11	17	82	1.21	1.58	1.87	0.32	0.45	0.48
12	24	85.5	1.88	1.69	1.98	0.59	0.5	0.55
13	3	93	2	1.8	2.10	0.52	0.55	0.62

GA, gestational age; hCG, human chorionic gonadotrophin; MoM, multiples of the median; PAPP-A, pregnancy-associated plasma protein A.
[a] Regression formulas: FB MoM $= 10^{(-0.1296+0.004103\times\text{GA days})}$, PAMoM $= 10^{(-0.87808+0.006593\times\text{GA days})}$. Regressed median at GA + 3 days for each week.
[b] Regressed values are from Evans *et al*. (2007).

Table 4—Standard deviation and correlation parameters of log10 MoM free beta hCG and PAPP-A distribution

			SD		
Dataset	Population	*N*	Free beta hCG	PAPP-A	R-value
Retrospective	Unaffected	1064	0.194	0.2383	0.2752
Retrospective	Down syndrome	54	0.2175	0.2505	0.2011
Routine screening	Unaffected	146 513	0.2086	0.2384	0.2648
Routine screening	Down syndrome	[a]	0.2306	0.2506	0.1973
Meta-analysis[b]	Unaffected	—	0.2655	0.2442	0.1890
Meta-analysis[b]	Down syndrome	—	0.2523	0.2736	0.0929

hCG, human chorionic gonadotrophin; MoM, multiples of the median; PAPP-A, pregnancy-associated plasma protein A.
[a] For the routine screening dataset, the Down syndrome parameters were determined by taking into account the difference in the unaffected parameters between the routine screening and retrospective datasets.
[b] Evans *et al*. (2007).

RESULTS

Free beta hCG medians decreased and PAPP-A medians increased between 9 and 13 weeks of gestation in control specimens (Table 2). In Down syndrome, free beta hCG was elevated and PAPP-A was decreased compared to unaffected pregnancies. The median MoM in Down syndrome cases, increased for both free beta hCG and PAPP-A with increasing gestation (Table 3). Table 4 shows the SD and correlation parameters for the Gaussian distributions from the retrospective dataset and then adjusted based on the routine screening data set.

Based on the Gaussian parameters from the retrospective dataset, the estimated detection rate at a fixed 5% false-positive rate using free beta hCG, PAPP-A and maternal age varied from 78% at 9 weeks of pregnancy to 70% at 13 weeks of pregnancy. Using a combined protocol with NT, the detection rate at a fixed 5% false-positive rate varied from 92 to 90% between 9 and 13 weeks of gestation. Adjusting the Gaussian parameters based on the routine screening data set reduced the detection rate by at most 1% (Table 5).

DISCUSSION

The data demonstrate that analysis of free beta hCG and PAPP-A using a dual analyte DBS assay is an effective tool in screening for Down syndrome. A key component of the effectiveness of a marker in Down syndrome screening is the SD. Based on the results in this study the SD of PAPP-A is similar to while the SD of free beta hCG is much smaller than that seen with other assays (Table 6). We attribute the tighter SD of free beta

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Table 5—Detection rate at a fixed 5% false-positive rate for free beta hCG and PAPP-A, with and without NT[a] at various gestational ages

Gestational age	Free beta hCG/PAPP-A	Free beta hCG/PAPP-A adjusted parameters	Free beta hCG/PAPP-A/NT	Free beta hCG/PAPP-A/NT—adjusted parameters
9	78	77	92	92
10	75	74	92	91
11	73	71	91	90
12	71	69	90	90
13	70	68	90	89

hCG, human chorionic gonadotrophin; NT, nuchal translucency; PAPP-A, pregnancy-associated plasma protein A.
[a] NT assumed to be measured at 12 weeks of gestation

Table 6—Free beta hCG and PAPP-A unaffected SD log10(MoM) in current study compared to other published studies

Free beta hCG				PAPP-A			
Rank	Publication	N	SD	Rank	Publication	N	SD
1	Current Study	1064	0.194	1	Brambati *et al*., (1994)	89	0.2214
2	Orlandi *et al*., (2005)	10 000	0.2177	2	Crossley *et al*., (2002)	16 953	0.225
3	Biagiotti *et al*., (1995)	246	0.2208	3	Spencer *et al*., (2002)/2003	12 781	0.2361
4	Forest *et al*., (1997)	500	0.2498	4	Tsukerman *et al*., (1999)	1564	0.237
5	Brambati *et al*., (1994)	89	0.2573	5	Current Study	1064	0.2383
6	De Biasio *et al*., (1999)	1454	0.26	6	Wheeler *et al*., (1998)	673	0.2415
7	Spencer *et al*., (2002, 2003)	13 073	0.2613	7	Avgidou *et al*., (2005)	30 234	0.2454
8	Wald *et al*., (2003)	425	0.2651	8	Wald *et al*., (2003)	425	0.2495
9	Wheeler *et al*., (1998)	673	0.2654	9	De Biasio *et al*., (1999)	1454	0.25
10	Avgidou *et al*., (2005)	30 234	0.2661	10	Haddow *et al*., (1998)	3169	0.251
11	Biagiotti *et al*., (1998)	200	0.2676	11	Casals *et al*., (1999)	267	0.2543
12	Muller *et al*., (2003)	5634	0.269	12	Orlandi *et al*., (2005)	10 000	0.2558
13	Crossley *et al*., (2002)	16 912	0.269	13	Wald *et al*., (1996)	377	0.2659
14	Weinans *et al*., (2005)	320	0.2727	14	Muller *et al*., (2003)	5636	0.267
15	Haddow *et al*., (1998)	3169	0.275	15	Biagiotti *et al*., (1998)	200	0.2868
16	Wald *et al*., (1996)	383	0.2833	16	Weinans *et al*., (2005)	320	0.2923
17	Tsukerman *et al*., (1999)	11 659	0.295	17	Forest *et al*., (1997)	500	0.3597
18	Casals *et al*., (1999)	136	0.3041				
19	Jauniaux (1996)	51	0.3474				
	Overall[a]	95 222	0.2648		Overall	85706	0.2441

hCG, human chorionic gonadotrophin; MoM, multiples of the median; PAPP-A, pregnancy-associated plasma protein A.
[a] Overall value determined by pooling, i.e. $\sqrt{\sum \left((N_i - 1) \times \mathrm{SD}i^2/(N - K)\right)}$, where N_i = the N of each individual study, and K = the number of studies).

hCG to the dried blood spot technology. The SD of any analyte represents a composite of the naturally occurring variation of the analyte in the population, assay imprecision and variability due to specimen handling and transport. In the routine screening part of our study, specimens represented a wide-spread cross-section of the US population and thus the naturally occurring variation component of the SD is likely to be close to its true value. With respect to the other two components of variation, assay imprecision for dried blood is higher but the variability due to specimen handling and transport is significantly less. Stability studies have shown increased levels of free beta hCG with increasing transportation time in liquid specimens. More significant may be the initial handling of the liquid specimen. With dried blood, whole blood is spotted onto filter paper and the drying process begins immediately. With liquid specimens, the specimen remains in its whole blood state until clotting and then separation of serum. Although a specimen is in its whole blood matrix, the amount of free beta hCG can increase significantly due to disassociation of total hCG compared to serum (Spencer *et al*., 1993). This increase is greater with higher temperatures. In routine screening, where specimens are collected from a large number of disparate sites, there is a lack of uniformity in the length of time and temperature at which the liquid specimen remains in its whole blood state and the subsequent time to analyses of post serum separation. Thus in the case of serum, the SD is impacted to a greater degree by the handling and transport time than with the dried blood spot technology.

Ultimately, overall screening performance is based not only on the SD of each marker but also on the mean of each marker and the covariance (correlation) between each pair of markers. In this study, free beta hCG Down syndrome means were not as extreme as in other studies, while PAPP-A Down syndrome means were more extreme (Table 3). In addition, the correlation in both the unaffected and Down syndrome distributions was higher in the current study (Table 4). To assess the

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Table 7—Mahalanobis distance for the combination of free beta hCG and PAPP-A

GA week	Current study	Meta-analysis
9	2.11	1.98
10	2.01	1.85
11	1.90	1.74
12	1.83	1.65
13	1.79	1.59

GA, gestational age; hCG, human chorionic gonadotrophin; MoM, multiples of the median; PAPP-A, pregnancy-associated plasma protein A.
Mahalanobis distance $= \sqrt{\boldsymbol{X}^T S^{-1} \boldsymbol{X}}$ where $\boldsymbol{X}$ is a vector of the mean log(MoM) and S^{-1} is the inverse of the 2×2 covariance matrix. The covariance matrix consists of the variance of each marker determined by squaring the average of the Down syndrome and unaffected standard deviation for each marker and the covariance between markers determined by multiplying the average of the Down syndrome and unaffected correlation by the average standard deviation for each marker.

ultimate capability of the combination of dried blood free beta hCG and PAPP-A to previous studies, we compared the Mahalanobis distance of the combination of free beta hCG and PAPP-A in the current study to the data in a previous meta-analysis (Evans *et al*., 2007). The Mahalanobis distance in this study was greater compared to the meta-analysis (Table 7), indicating that the combination of free beta hCG and PAPP-A worked better in dried blood compared to previous meta-analysis. Thus, the tighter SD of free beta hCG, the lower means of PAPP-A and the higher correlation between free beta hCG and PAPP-A outweigh the somewhat less extreme Down syndrome free beta hCG means.

A review of the literature showed that free beta hCG was a superior marker to intact hCG in first trimester screening (Evans *et al*., 2007). A second review (Palomaki *et al*., 2007a) also showed improvement with free beta hCG with this improvement greatest at 11 weeks although the authors claimed that the markers were interchangeable. The same authors with other colleagues also published a comparison study of free beta hCG vs intact hCG (Canick *et al*., 2006); however, the unaffected SD of free beta hCG in that study was significantly greater than that seen in the literature and would have been considered an outlier if it had been included in the authors review study. In any case, independent assessments of the literature worldwide has led to most centers using free beta hCG instead of intact hCG in first trimester screening, predominantly with liquid blood sampling. The current study along with previous population-based studies on the use of dried blood spots in first trimester screening (Orlandi *et al*., 1997; Krantz *et al*., 2000; Wapner *et al*., 2003; Orlandi *et al*., 2005) have evaluated free beta hCG along with PAPP-A. Another study (Palomaki *et al*., 2007b) demonstrated that intact hCG does not work as well in dried blood spots as in liquid serum. As a result, intact hCG cannot be considered a viable option when using dried blood spot technology and those choosing to use dried blood spot technology should do so with free beta hCG.

One concern with the study is that in the retrospective sample set there were only 12 specimens at 9 weeks of gestation and the observed median for free beta hCG was much greater than the regressed median. However, one of the reasons regression is used is that the pattern established with data at other weeks can be used in addition to the data solely collected at the given gestational age. Such an approach has been used extensively in Down syndrome screening. In the routine screening study set we had 3882 patients at 9 weeks and the median MoM in this group was 0.95. Therefore, we believe the estimates of screening performance at 9 weeks of gestation are reasonable.

DBS technology offers a number of logistic advantages in addition to the analytical advantages. First, unlike with liquid blood specimens the dried blood specimen can remain attached to the requisition form thus ensuring the integrity of patient identification. Second, elimination of liquid specimens inside glass test tubes reduces the biohazard risk and greatly reduces the packaging and associated transportation costs. Third, specimens may be collected *via* fingerstick, thus obviating the need for a phlebotomist. As a result, the technology may be disseminated to remote areas where typical venipuncture collection is not available. Thus, dried blood spot technology adds.

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