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Detection of Immunoglobulin Isotypes from Dried Blood Spots

Nancy J Andersen^{1,*}, Tapan Kumar Mondal^{1,*}, Mark T. Preissler², Brian M. Freed³, Sabine Stockinger³, Erin Bell⁴, Charlotte Druschel⁵, Germaine M. Buck Louis⁶, and David A. Lawrence¹

¹Wadsworth Center, New York State Department of Health, Albany, NY 12208

²Serology/Immunology Lab, Albany Medical Center, Albany, NY 12208

³University of Colorado, ClinImmune Labs, Aurora CO 80045

⁴University at Albany School of Public Health, Rensselaer, NY 12144

⁵Congenital Malformations Registry, NYSDOH

⁶Division of Intramural Population Health Research, Eunice Kennedy Shriver National Institute of Child Health & Human Development

Abstract

The study was designed to determine the sensitivity and reproducibility of recovering immunoglobulin (Ig) isotypes (IgG subclasses, IgA, IgE and IgM classes) from dried blood spots (DBS), a methodologic subcomponent of the Upstate KIDS Study. A multiplexed Luminex assay was used for IgG1/2/3/4, IgA and IgM analysis; an ELISA was used for IgE. Plasma samples from de-identified patients were used to compare the Luminex assay with nephelometry, which is routinely used to quantify IgA, IgG and IgM in clinical samples. The IgE ELISA was compared to an immunofluorescence assay. Prior to evaluation of punches from newborn dried blood spots (NDBSs), recoveries of Ig from punches of cord blood DBSs (CBDBSs) vs. plasma from the same cord bloods were compared. Although the recoveries of Ig from plasma and DBSs were not comparable, which could be due to cell lysates in the DBS samples, the analyses were reproducible. Additionally, the levels of IgA, IgG2, IgG4, and IgM recovered from CBDBSs positively correlated with those in plasma. The DBS data is a relative value since it is not equivalent to the plasma concentration. The majority of Ig concentrations recovered from 108 newborns of the Upstate KIDS Study were within the range of newborn plasma Ig levels with the exception of IgG3. The IgG4 values displayed the greatest variance with a wide range (0.01–319 mg/dl), whereas, IgG1 values had the narrowest range (85.2–960.4 mg/dl).

Address correspondence to: Dr. David A. Lawrence, David Axelrod Institute, 120 New Scotland Ave, Albany, NY 12208
David.Lawrence@Wadsworth.org.

*Equal contributors to this study

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1. Introduction

Eluted proteins from newborn dried blood spots (NDBS) have been used to assess the levels of cytokines and chemokines; however, to date, few studies have quantified the levels of immunoglobulins (Igs). Most likely, there have been few studies of newborn Ig levels, because most antibodies are of the IgG class, and maternal IgG antibodies are transported through the placenta. Previously, only the four IgG isotypes (IgG1/2/3/4) were suggested to be transported through the placenta. The exclusive transport of the IgG subclasses from mother to infant has recently been challenged with the investigation of immunodeficient babies with maternal IgA (Borte et al., 2012). The quantities of each of the Ig isotypes, including the IgG subclasses, regardless of whether a portion of the Ig is from the mother, could provide clues about developing disorders or altered processes.

The IgG subclasses are not equally transported through the placenta by the neonatal Fc receptor (FcRn) (Palmeira et al., 2012), in that usually IgG1 displays the greatest transport and IgG2 the lowest (Costa-Carvalho, et al., 1996). IgG transfer to the fetus increases in the third trimester with the greatest amounts occurring in the last 4 weeks of gestation (Saji et al., 1999). Interestingly, it has been suggested that all IgG subclasses are transferred with reduced efficiency into at term neonates with a low birth weight, but IgG1 and IgG2 subclasses are transferred with significantly less efficiency than IgG3 and IgG4 (Palmeria et al., 2012).

All of the Ig isotypes have different biological activities (Schur, 1987; Ravetch and Kinet, 1991; Hamilton, 2001), including activation of complement and binding to specific Fc receptors on different cell types via their C-terminal (Fc) domain. IgG isotypes bind to different FcγRs (FcγRI, CD64; FcγRII, CD32; and FcγRIII, CD16). The FcRs of leukocytes have different affinities for the Ig isotypes; IgG1 and IgG3 bind with higher affinities than IgG2 and IgG4. Neutrophils bind IgA better than the other types of leukocytes (Lawrence et al., 1975). Human basophils, eosinophils, mast cells, neutrophils, monocytes, macrophages, dendritic cells, Langerhans cells, and platelets have a high-affinity receptor for IgE (FcεRI) (Kinet, 1999); whereas, low-affinity IgE receptors (FcεRII or CD23) are on lymphocytes (Lawrence et al., 1975; Conrad, 1990; Acharya et al., 2010). Both FcεRI (Untersmayr et al., 2010) and FcεRII (Kaiserlian et al., 1993) are expressed by intestinal epithelial cells and have been implicated in enteropathies. High levels of IgE usually reflect atopy or infections with parasites, such as *Schistosoma*. Although *in utero Schistosoma* infection leads to elevated cord blood IgE levels (Seydel et al., 2012), high levels of cord blood IgE as a predictor of later allergies or asthma has been controversial. Prenatal stress has been reported to increase the amount of IgE in cord blood (Peters et al., 2012). Environmental exposure to the immunotoxicant lead also has been linked to elevated levels of cord blood Pb levels (Annesi-Maesano et al., 2003). Lead preferentially enhances the activity of Th2 cells, which promotes IL-4 and IgE production (Heo et al., 1996); prenatal lead exposure of mice enhanced neonatal serum IgE levels (Snyder et al., 2000).

Fetal B cells and T cell exist by the second trimester, suggesting humoral immunity may begin early in development if antigens or immunomodulators, such as lead, are presented, and fetal B cell clones may have skewed synthesis of IgG1, 2, 3, and 4 or the other isotypes

with prenatal exposures. Thus, aberrant proportions of the isotypes could be predictors of unusual prenatal conditions that could increase the prevalence of disorders/diseases as the child develops. The early levels of the Ig isotypes may also shed light on other aspects of the fetomaternal relationship. Elevated levels of IgA or IgM could indicate that the protective germ-free environment has been compromised. Since IgM does not cross the placenta, neonatal IgM levels could be used to determine prenatal and early postnatal infections. IgM from DBSs has been used to detect antibodies to *Toxoplasma gondii* (Mei et al., 2011) and measles (Uzicanin et al., 2011). Neonatal IgG4 levels usually are low, because placenta transport is not efficient and maternal IgG4 levels are usually lower than the other IgG isotypes. Recently, IgG4 has been associated with sclerosing disease and other diseases (Stone et al., 2012); elevated levels of IgG4 could suggest unusual early immune responses. An abnormal increase in any isotype could relate to a lymphoma and aberrant immune activation.

This report describes the methods used to quantify the Ig levels from NDBSs and how these levels compare to those in cord blood. For the analysis of cord blood, the Ig concentrations of cord blood plasma and those eluted from dried cord blood of the same baby were compared. The rationale for undertaking this work was to determine if NDBSs could be used to characterize infants' immunologic status, and also for assessing in utero infection as a potential confounder of the relation between infertility treatment and infant's growth and development through three years of age. This latter purpose is the central research goal of the Upstate KIDS Study [www.albany.edu/sph/upstatekids.php]. The quantitative differences of measured Ig obtained from the various samples are discussed with regard to protein recovery and free vs. bound proteins.

2. Material and Methods

2.1 Patients and samples

De-identified plasma or anonymized cord blood was provided by Dr. Mark Preissler or Dr. Brian Freed, respectively. Anonymized punches (3.2 mm) from NDBSs were obtained from the Newborn Screening Program of Wadsworth Center, NYS Department of Health under an exempt protocol (NYS IRB # 07-070); a 3.2 mm punch, which is the standard size for all Newborn Screening Programs, allows the most efficient number of punches/DBS (9 punches/DBS). De-identified punches from 108 participants of the Upstate Kids Project were obtained with written informed consents of a non-exempt protocol (NYS IRB # 07-097). The amount of blood contained in a 3.2 mm punch was calculated to be 3.275 μ l (Fig. 1). Estimating that 50 % of the blood is plasma, we calculated that each punch would therefore contain 1.6375 μ l of plasma.

2.2 Sample preparation methods

The different preparations of each collected cord blood (CB) were processed and shipped overnight. The CB was first collected with a 16 gauge ultra thin wall needle into a 250 mL sterile Blood Pack Unit containing 35 mL of the anticoagulant Citrate Phosphate Dextrose Solution USP (CPD; Fenwal, Cat # 4R0837MC). For the collection of plasma, a portion of each CB was centrifuged (655 x g for 10 min at 4°C), and the plasma was then immediately

frozen. For the preparation of the cord blood dried blood spots (CBDBS), 50 μ l of CB was spotted five times onto a Guthrie card, then dried and sealed in a plastic bag with desiccant. All punches of CBDBS were maintained at -20° C until beginning sample analysis.

2.3 Reagents

For multiplexed analysis of the IgG subclasses, IgM, and IgA, the Milliplex[®] magnetic bead panel for human immunoglobulin isotyping (HGAMMAG-301K-06, EMD Millipore Corp., Billerica, MA) was used. For the IgE analysis, an ELISA was used (Cat # 3810-1H-20, MABTECH Inc., Cincinnati, OH). The ELISA kit for IgE contained mAb107 capture antibody, mAb182-biotin detection antibody, streptavidin-HRP, and the IgE standard. The substrate used was TMB (Sigma, Cat # T 4444).

2.4 Quantification of IgA, IgG and IgM

Both CBDBS and NDBS were extracted overnight (18–20 hr) at 4° C with 60 μ L of elution buffer in low-binding 96-well round bottom plates (catalog #1830-9600, USA Scientific, Ocala, FL) on an orbital shaker (Titer Plate Shaker, model # 1830-9600, USA Scientific, Ocala, FL) at 500 ± 50 rpm. The elution buffer was comprised of Phosphate Buffered Saline (PBS, Lonza catalog #17-516Q) with 0.1% BSA (Sigma-Aldrich, catalog #A-4503) and EDTA-free protease inhibitors (1 tablet/10 ml, Roche Applied Science, catalog #04693159001, Mannheim, Germany). Following extraction, the eluants were frozen at -80° C until analysis was performed. The analysis protocol followed the manufacturer's magnetic bead panel instructions with one exception: the CBDBS and NDBS eluants were run at a 1:10 dilution for IgA, IgG1, IgG2, and IgM, and at a 1:250 dilution for IgG3 and IgG4. This was accomplished by incubating the dilutions separately with the respective beads, and then recombining all the respective samples together after the capture antibody step. All samples were diluted with PBS containing 0.1% BSA and gently vortexed (Thermolyne, Maxi Mix II) prior to the incubation. The diluted samples were incubated on an orbital shaker (500 ± 50 rpm; Titer Plate Shaker, Thermo Scientific) with the capture-antibody coated beads for 2 hr at room temperature (RT). The plates were then washed on an ELISA washer with a magnet for capturing the beads (BioTek, ELx405 Select CW), and incubated with the detection solution. Analysis was accomplished using a Luminex¹⁰⁰ analyzer (Luminex System, Austin, TX) with xPONENT 3.1 software (Luminex). The LODs for the six isotypes were IgG1, 55 ng/ml; IgG2, 85 ng/ml; IgG3, 0.6 ng/ml; IgG4, 0.9 ng/ml; IgA, 5.7 ng/ml; and IgM, 7.5 ng/ml. The results of this analysis were compared to those performed by the Serology Lab at Albany Medical Center for IgA, IgM and total IgG; these results were obtained with nephelometry using a Beckman IMMAGE 800.

2.5 Quantification of IgE

Due to the low amounts of IgE in extracted CBDBS and NDBS samples, CBDBS or NDBS punches were extracted individually in wells of a high-binding ELISA plate (Costar, Cat # 3369) that was pre-coated with anti-IgE capture antibody mAb107. The IgE ELISA wells were coated with 50 μ l/well of mAb107 (2 μ g/ml PBS), and the plate was incubated at 4° C overnight. The plate was then washed twice with PBS and blocked with 200 μ l/well of Blocking/Washing buffer (PBS containing 0.05 % Tween-20 (Sigma, Cat # P-1379) and 0.1% BSA) for 2 hr at RT, then washed six times with wash buffer. For the CBDBS or

NDBS IgE analysis, 50 µl of MABTech assay buffer was added to each well; punches spotted with either IgE standard or those collected from the CBDBS or NBDBS card were then added to their respective wells. To create the IgE standard punches, the standard curve was prepared according to the manufacturer's instructions in low-protein binding microfuge tubes. Punches were then prepared from blank Guthrie cards and placed into a 96-well plate. The standard IgE punches were made by adding 3 µL (the volume that saturated the punch) of individual IgE standards (0.156–10 ng/ml). These IgE-spotted punches were kept at RT for 2½ hr and then stored at –20°C until use. For the assay, the standard curve was generated using duplicate punches, while the sample punches were analyzed in singlicate. For the blank, no punch was placed in duplicate wells. The anti-IgE coated plate was incubated with the punches overnight at 4°C with shaking on the titer plate shaker (500 ± 50 rpm). Following the capture antibody step, the punches were removed and the plate washed six times. For the plasma IgE assay, 50 µl of plasma was added to the appropriate wells; the protocol for the plasma incubation thereafter followed the manufacturer's instructions (MABTECH Inc).

For both assays, 50 µl/ well of detection antibody anti-IgE biotin mAb182 (0.3 µg/ml) was added to each well. The plate was then incubated for 2 hr at RT and washed 6 times. Streptavidin-HRP (50µl/well) was then added to each well at a concentration of 1:1000 in blocking buffer) and incubated for 1hr at RT. The washes were repeated, and TMB substrate was added (20–30 min in the dark at RT followed by 25 µl/ well of 1M H₂SO₄ stop solution). The plate was then immediately read at 450 and 630 nm on an EL808 reader and analyzed with KC4 software (BioTek, Winooski, VT). The LOD for the IgE ELISA was 0.1 ng/ml. These results were compared to an automated fluorescent EIA performed using the Phadia ImmunoCAP system (LOD of 2U/ml or 4.76 ng/ml). To determine the IgE level, a Lymphochek® Immunoassay Plus Control (Bio-Rad) was used.

2.6 Immunoprecipitation and SDS gel analysis

Capture antibody labeled beads from the Ig kit, or an anti-IgE coated well, were used to capture the Ig isotypes. The anti-IgA, anti-IgG (pooled subclasses), and anti-IgM beads were separately incubated with the eluants as described. After the beads or wells were washed, detection antibody was not added. The captured analytes were eluted at 70°C for 10 min with 10% SDS buffer containing 10 mM 2-mercaptoethanol; beads and wells without the addition of the eluants were also assayed on a 10% acrylamide gel. Separate lanes were analyzed by Western blot using HRP-goat anti-Ig (goat anti-human IgG, IgM, IgA horseradish peroxidase (HRP) conjugate, Millipore Cat # AP120P), as previously described (Lawrence et al., 2007).

2.7 Statistical analysis

Analysis of the data was performed using software as previously described, and descriptive statistical analysis was performed with GraphPad Prism 4.0 software. Paired t-test analysis was used for data matched individually (Table 5); the unpaired t-test determined the p values for data pairs when the data was unmatched (Table 6). Correlation analysis was performed using the Pearson correlation analysis (Figures 2 and 3), and outlying data points from the NBDBS Ig results (Fig. 5) were obtained via Grubb's test (Taylor, 1990).

3.0 Results

3.1 Comparative analysis of methods to quantify Ig isotypes from plasma samples

The plasma Ig concentrations of 15 leftover de-identified patient samples were assayed by both nephelometry and a Luminex assay. In general, the Ig values were higher with the Luminex technology (Table 1). The mean $\pm$ SEM percent differences and the range for IgA, IgM and IgG were 96.9 ± 17.1 and 47–322%; 61.9 ± 3.7 and 36–82%; and 68.8 ± 6.8 and 32–120%, respectively. The correlations from two separate runs were combined and are shown (Fig. 2). Correlations for IgA and IgM were good, but the correlation for IgG was low, possibly because total IgG was measured by nephelometry while in the Luminex assay each of the subclasses were measured separately. These values were then summed to determine the total IgG value. Ten leftover clinical samples were also assayed for IgE as previously described; the ELISA method provided consistently lower values than the fluorescent EIA method (Table 2). While the ELISA results were only 55.3 ± 8.8 % (range 1.4–80%) of the EIA values overall, the correlation between the two assays was good ($r^2 = 0.99$, Fig. 2).

3.2 Reproducibility of the Luminex and ELISA analysis of Ig extracted from the CBDBS punches

In order to assess reproducibility among punches, Ig was eluted from five separate CBDBS punches. Pooled eluants from the punches were each assayed ten times to assess intra-assay coefficient of variation (%CV); this analysis was repeated a second time to assess inter-assay differences (Table 3). The %CVs ranged from 2.02 to 28.1; the overall average %CV was 8.6. The eluted samples were assayed for IgE as well; no measurable IgE was detected using the fluorescent EIA. Ten punches from 3 separate CBDBS donors were assayed; each CB was spotted five times on a card and punches were taken from the CBDBSs. The ELISA used IgE standards eluted from punches for quantification, but this assay protocol does not allow the analysis of pooled samples as individual punches are eluted in anti-IgE coated wells. Thus, due to the nature of the assay, the variances for IgE are in actuality an inter-assay comparison. The %CVs were much higher for these IgE results since the values are obtained from different CBDBS; the mean $\pm$ SD and %CVs were 1.60 ± 0.55 , 34.4; 1.58 ± 0.52 , 33.0; and 1.2 ± 0.78 , 65.1.

3.3 Influence of punch location on recovery of Ig from CBDBS

Since the Ig is eluted from a 3.2 mm punch taken from a dried blood spot that is approximately 12.5 mm in diameter (Fig. 1), we determine whether the Ig concentrations would vary dependent on where the DBS is punched. Although usually only spots that have uniformly distributed blood are punched, it is possible that Ig in the blood does not distribute and adsorb equally throughout the spot. To evaluate intra-spot differences, six punches were taken from different regions of a CBDBS from four different CBs (Table 4). The average %CV for all Ig was 19.29 ± 2.16 (mean $\pm$ SEM); the highest average %CV was for IgG3 (23.5%). Since this variation would include differences with regard to elution and assay differences as well as intra-spot differences, the overall variation among punches was relatively low.

3.4 Comparative amounts of Ig in CB plasma and Ig recovered from the CBDBS punches

The Ig isotypes recovered from the CBDBS punches were not comparable to the isotype concentrations of the plasma. IgG1, IgG2, and IgM results from CBDBS were lower than those from matched plasma samples; whereas, IgA, IgG3, IgG4, and IgE were higher (Table 5). The serum isotype concentrations recovered from the CBDBS punches were significantly different for all isotypes except IgG4. The Ig isotype concentrations in plasma and recovered from the CBDBS punches correlated for IgA, IgG2, IgG4, and IgM (Fig. 3). Significant correlations did not exist for IgG1 ($r^2 = 0.01$, $p=0.58$), IgG3 ($r^2 = 0.002$, $p=0.79$), and IgE ($r^2 = 0.05$, $p=0.42$).

Interestingly, however, for the CB plasma and CBDBS comparisons of IgM and IgA, the CB plasma was initially used at a 1:2 dilution. At this dilution the IgM plasma values were nondetectable, which was surprising as detectable values were obtained from the CBDBS punches. However, taking into account the overall dilution of the plasma contained on each punch, the punch eluants had a final dilution of 1:366. The plasma was thereafter run at dilutions of 1:4, 1:40, 1:400, and 1:4000; the mean IgM values $\pm$ SD from 7 different CB plasma samples were 299 ± 124 , $11,681 \pm 4,482$, $53,692 \pm 25,600$, and $63,150 \pm 30,210$ ng/ml, respectively. Similar interference of IgG subclass analysis was also observed at dilutions $<1:400$. Although similar interference may exist for measurement of IgA, this could not be determined as detectable values could not be obtained when increasing the plasma dilution values.

3.5 Immunoprecipitation and Western blot analysis of Ig recovered from CBDBSs

CBDBS punches were processed as described in the Methods section. The anti-IgG beads captured the most protein, likely as the IgG levels are higher than IgA and IgM. Therefore, the IgG lane shows the densest bands (Fig. 4). In the IgG lane, the densest bands were confirmed to be the heavy and light chains by Western blot analysis (not shown). The high molecule band, a barely visible band at about 40 kDa, and a lower MW band than the light chain band did not react with the HRP-goat anti-Ig. The IgM lane also had a band at about 35 kDa and a band of lower MW than the light chain that did not react with the anti-Ig. No bands were detected for IgE, which likely is due to its low concentration and the heat lability of IgE (Mota, 1964). The IgG and IgM results suggest that other serum proteins may be captured with IgG and IgM, although it is possible that the extra bands are Ig fragments.

3.6 Relative amounts of Ig isotypes from NDBS punches

The concentrations (mean $\pm$ SEM) of IgM, IgG1, IgG2, IgG3, IgG4, and IgA eluted from the NDBS punches were 3.3 ± 0.2 , 309 ± 13 , 84.5 ± 5.1 , 35.5 ± 2.7 , 22.6 ± 4.5 , and 1.7 ± 0.2 mg/dl, respectively. The IgE concentrations eluted from the NDBS punches were 3.49 ± 0.24 ng/ml. Although only the mean value for the IgG3 was above the normal serum range, some sample results fell outside of the normal range (Fig. 5). None of the NDBS samples had IgA or IgM concentrations beyond the normal ranges for plasma samples. However, IgG1, IgG2, IgG3, IgG4, and IgE had 30, 21, 0, 14, and 0 samples below their ranges and 4, 9, 62, 19, and 21 samples above their respective ranges. Interestingly, IgG4 values had the greatest range (30,664-fold), and IgG1 values had the lowest range (11.3-fold). The Ig isotype concentrations from the NDBS punches were compared for outliers, as described in the

Methods section. The numbers of outliers for IgA, IgE, IgG1, IgG2, IgG3, IgG4, and IgM from the 108 NDBS punches were 2, 3, 1, 0, 2, 6, and 2, respectively. For 33 CBDBS punches, IgG4 also had the most outliers with 5.

3.7 Comparison of Ig concentrations from CBDBS and NDBS punches

Since the CB was immediately spotted onto a Guthrie card upon isolation, and the NDBS was made with a drop of blood from a baby's heel within the first few days after birth, the Ig values were compared to determine if a significant difference might exist for a particular Ig isotype. Even though the CBDBS and NDBS are from different individuals, it was deemed possible that Ig concentrations could be used to assess if blood from babies vs. from CB might differ, thus reflecting influences of the newborn on the distribution/synthesis of Ig. Although all of the IgG subclass concentrations recovered from the NDBS punches were greater than those from the CBDBS punches, only IgG1 and IgG3 were significantly greater (Table 6). The IgA and IgM levels did not differ. The IgE levels from the NDBS punches were significantly greater than those from the CBDBS punches. Although the differences may simply be due to the particular demographics of the sample sources, it is possible that cord blood and systemic blood from babies may have different levels of these isotypes.

4.0 Discussion

4.1 Methodological comparison assays

Ig isotypes from clinical samples were first assayed to assess how the Luminex and ELISA methods used for this study compared to the methods validated by a local medical center. There was a good correlation between the methods, even though for the IgG levels the methods were very different; in that the calculation of a total IgG concentration from the individual subclasses was summed from the Luminex assay results. There is also the possibility that differences in the characteristics of the reagent antibodies in the two assays may have affected quantification of IgG. Assay differences due to the specificities and affinities of antibodies have been previously reported and are factors when comparing results with reagents from different manufacturers (Aziz et al., 1999; Perry et al., 2009).

Interestingly, for IgE analysis, the immunofluorescent EIA was more sensitive than the ELISA for plasma samples. However, for analysis of the CBDBS punches, only the ELISA method was able to give detectable values. This difference may reflect the lability of IgE, in that it is the most labile of all Ig isotypes, especially to heat (Mota, 1964). The ELISA method directly captured the IgE as it was being eluted in the cold; whereas, the EIA used samples that were eluted from the CBDBS punches and then frozen until assessment. It is possible that immediate capture may help to stabilize the IgE structure. Another possibility for the difference is that immediate capture of IgE upon elution from the punch could lessen interference from other eluted constituents, including those from cell lysates, such as the high and low affinity FcεRs.

The lower concentration of the Ig isotypes from the punches of the CBDBSs may also reflect the lability of the other isotypes as discussed for IgE. Although IgG is usually considered a relatively stable protein, to our knowledge, there has been no prior quantification of blood IgG levels after the blood was dried onto paper. Besides the stability

of the Ig isotype epitopes, which are needed for capture and detection, interference from FcRs being released from leukocyte lysates also could affect the analysis. Thus, the Ig values may represent only free Ig and not Ig-FcR complexes. However, the immunoprecipitation and Western blot analysis of the isotypes eluted from the CBDDBS punches suggest that if other proteins are associated with the Ig they are at a minimum, in that protein bands other than the heavy and light chains were barely visible.

4.2 Differences between CBDDBS and NDBS punches

Although the differences in recovery of Ig from the CBDDBS and NDBS punches may simply be due to the particular demographics of the newborns, it is possible that cord blood and systemic blood from babies may have different levels of these isotypes. Interestingly, the NDBS punch levels of IgE were significantly greater than those of the CBDDBS punches; this suggests birth with the exposure to antigens and early cytokine production of IL-4, IL-5, and IL-13, may promote Ig class switching. Cytokine involvement in early IgE levels has been reported in many studies (Crestani et al., 2007; Neaville et al., 2003; Herberth et al., 2013), but involvement in later development of asthma has been controversial (Rothers et al., 2011; Ohshima et al., 2002; Williams et al., 2000). Additionally, isotype-restricted responses dependent on Th1 vs. Th2 cytokines have been reported to be dependent on the haplotype of the newborns (Jafarzadeh and Shokri, 2012). Besides IgE, the IgG subclasses are differentially influenced by Th1 and Th2 cytokines; IgG4 seems to be the only IgG subclass preferentially promoted by a Th2 response. Although IgG4 has been implicated in atopic asthma and allergies (Jenmalm and Bjorksten, 1999), more recently it has been suggested to modulate allergic responses (Platts-Mill et al., 2001; Kihlstrom et al., 2005). When the IgG1:IgG4 ratio decreases, there is greater tolerance to allergens (Scott-Taylor and O'B Hourihane, 2010). Since all of the IgG subclasses can respond to allergens and non-allergens, their biological influences need to be carefully considered. IgG4 is an especially interesting IgG in that it has low complement activation capacity and low binding to Fc γ Rs. IgG4 has unique structure and biology (Nirula et al., 2011), and recently has been implicated in various diseases and autoimmunities (Stone et al., 2012). As noted from the NDBS data (Fig. 5), our results with newborn blood already confirm the report that IgG4 is the most variant of the IgG subclasses with regard to its concentration; it has been reported that adult IgG4 serum levels range from 1–140 mg/dl (Nirula et al., 2011).

4.3 Potential for the use of DBS punches in future analyses

As discussed, the IgG levels of newborns mainly reflect the IgG concentrations of their mothers. However, subtle differences may exist due to passage through the placenta and the health of the mother and the newborn. Thus, the concentrations of the Ig subclasses and classes may be useful predictors of later health even though antigenic stimulation of newborn immunity and cytokine influences due to environmental exposures are only beginning. The collection of NDBSs as well as DBSs in general is considered an extremely value tool for future epidemiological investigations. These samples require only a drop of blood, which allows easy collection in the field. Furthermore, the Ig values appear to be relatively stable after collection although the concentrations may reflect additional parameters that need to be considered. Cell-associated modifications to the final levels may cause the DBS values to differ from plasma values. The B cell levels could enhance the

levels due to lysis and release of their Ig. The release of FcRs from leukocytes could block epitopes of Ig and inhibit Ig values. Regardless of complications that could alter the DBS Ig values, the results from 108 newborns of the Upstate KIDS Study are very consistent; there were very few outliers even for the most variant Ig.

5. Conclusions

Our findings suggest that NDBSs offer promise for population based research designed to answer etiologic questions about preconceptional or in utero exposures and infants' health status, as globally defined. The detection of outliers might be informative of possible immunological differences among select infants.

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Highlights

- Immunoglobulins (Ig) of all isotypes are reproducibly recovered from dried blood spots.
- The mean values of the Ig isotypes recovered from punches of newborn dried blood spots (NBDBS) were within the range of plasma Ig isotypes except for IgG3 which was greater.
- The IgG4 levels from punches of NDBS varied the greatest and IgG1 the least

Figure 1.

The Ig was extracted from punches taken from a dried blood spot on a Guthrie Card (A). A hanging drop of 50 µl of cord blood was absorbed onto an encircled area and a 3.2 mm punch is taken (B).

Figure 2.

Correlation analysis of methods to assess Ig isotypes. IgA, IgM, IgG analysis by nephelometry vs. Luminex and IgE by an immunofluorescence assay vs. ELISA were compared using plasma samples. The methods showed significant positive correlations.

Figure 3.
Correlation analysis of CB plasma Ig levels and the Ig concentrations recovered from the CBDBS.

Figure 4. SDS-PAGE of proteins eluted from Milliplex anti-IgA, anti-IgM, and anti-IgG beads and anti-IgE wells. The arrows indicated that the bands were identified by Western blot analysis to be the heavy (H) and light (L) chain bands of IgM, IgG and IgA. The arrow heads indicate the bands that did not react with goat anti-human IgA/IgG/IgM.

Figure 5.

Ig recovered from NDBS. Ig isotypes eluted from 108 NDBSs were assayed by Luminex for IgA, IgM, and IgG subclasses (mg/dl) and by ELISA for IgE (ng/ml). The levels and ranges (log scale) are reported in relation to clinical plasma ranges for these Ig classes and subclasses. The boxes represent the 25th and 75th percentiles and the median values; the extended lines are the 10th and 90th percentile, and the dots are the samples beyond these percentiles, the outliers for this analysis.

Table 1

Immunoglobulin Concentrations (mg/dl)

Specimen	IgA	IgM	IgG1	IgG2	IgG3	IgG4	IgG*
1	206 (146)	424 (228)	912	513	87	<1	1512 (751)
2	51 (24)	87 (31)	940	209	168	5	1322 (416)
3	275 (166)	114 (69)	1151	1127	75	170	2523 (1220)
4	163 (114)	130 (81)	1384	412	98	11	1905 (1120)
5	43 (25)	>500 (3710)	230	431	45	27	733 (368)
6	55 (172)	43 (23)	1139	214	57	32	1442 (647)
7	355 (202)	429 (204)	2320	64	159	344	2887 (1310)
8	95 (73)	188 (86)	1180	254	79	44	1557 (1170)
9	222 (139)	291 (218)	1470	282	157	0.7	1910 (2060)
10	302 (294)	142 (85)	630	163	92	30	915 (850)
11	91 (93)	52 (34)	1460	340	47	852	2699 (2290)
12	40 (41)	16 (11)	335	106	15	5.3	461 (396)
13	50 (45)	119 (98)	530	216	75	136	957 (837)
14	245 (277)	83 (66)	1042	411	88	2.5	1544 (1850)
15	25 (31)	13 (10)	222	120	19	nd	361 (174)
Adult Range	64–395	48–271	422–1292	117–747	41–129	1–291	695–1547

Value calculated as mean from two dilutions of serum assayed in duplicate with Milliplex kit and Luminex assay. The value in parenthesis is AMC value performed with nephelometry; nd=not detectable;

* IgG Luminex values were summed from the IgG subclasses.

Table 2

Plasma IgE analysis by EIA and ELISA (ng/ml)

Sample	EIA ^a	ELISA	ELISA:EIA Ratio
1	2606.4	2000	0.767
2	6249.6	4408	0.705
3	157.68	48	0.304
4	271.2	102	0.376
5	32.16	10	0.311
6	1636.8	1245	0.761
7	<4.76 ^b	1.9	~0.399
8	3710.4	2590	0.698
9	775.2	620	0.800
10	9.36	1.0	0.11

^aThe EIA assay reports values as U/ml with LOD of 2U so results are converted (U~2.38 ng) so that they would be comparable to ELISA.

^bThe EIA value was <2U/ml

Table 3Intra- and Inter-assay evaluation of the Luminex analysis of CBDDBS^a

Ig isotype	Plate	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Mean
IgM	1	4.34 ^b	9.28	4.59	4.53	2.02	4.95 ^c
	2	7.03	8.30	4.80	15.99	2.87	7.80
IgG1	1	4.11	15.06	7.69	4.93	4.42	7.24
	2	8.47	9.83	5.93	4.34	2.75	6.26
IgG2	1	4.09	5.40	3.53	5.70	3.35	4.41
	2	3.81	6.86	4.63	28.10	2.92	9.26
IgG3	1	15.59	21.69	14.15	9.92	5.19	13.31
	2	18.63	19.79	13.62	9.25	4.47	13.15
IgG4	1	13.91	9.92	12.01	9.51	7.64	10.60
	2	12.57	12.53	14.69	13.85	7.76	12.28
IgA	1	17.80	4.28	2.66	5.73	3.26	6.75
	2	19.92	4.01	2.55	4.22	2.72	6.68

^a CBDDBS punches from five CBs were eluted, pooled, and assayed 10 times (intra-assay) on two separate days (inter-assay).^b %CV;^c Mean %CV for 5 samples/plate/day

Table 4

Variance of Ig (mg/dl) recovery from CBDBS dependent on punch location

Specimen	IgM	IgG1	IgG2	IgG3	IgG4	IgA
A	Avg	102.150	34.990	21.032	0.509	0.063
	Stdev	10.341	3.796	5.843	0.085	0.011
	%CV	10	11	28	17	17
B	Avg	84.380	50.770	12.626	3.453	0.028
	Stdev	17.442	10.211	5.521	1.490	0.005
	%CV	21	20	44	43	16
C	Avg	108.630	28.970	3.726	0.451	0.032
	Stdev	23.132	3.431	0.406	0.048	0.003
	%CV	22	12	11	11	10
D	Avg	66.730	34.250	3.015	1.248	0.032
	Stdev	10.433	14.222	0.318	0.226	0.008
	%CV	24	42	11	17	25

Table 5

Plasma Ig versus Ig recovered from DBS of cord blood (mg/dL)

Immunoglobulin	CB Plasma			CBDBS			%Recovery ^b			p ^c
	Mean ^a	Stddev	%CV	Mean	Stddev	%CV	Mean	Stddev	%CV	
IgM	6.5	3.8	57.8	3.8	2.5	66.5	59.9	15.9	26.7	0.0008
IgG1	356.6	76.2	29.4	259.3	76.2	29.4	78.8	25.8	32.8	0.0003
IgG2	114.8	76.9	66.9	77.9	50.6	64.9	71.1	17.5	24.6	0.02
IgG3	9.1	6.3	69.5	19.1	16.5	86.2	291.3	344.2	118.2	0.062
IgG4	11.2	9.4	84.3	12.3	15.6	127.1	110.5	100.1	90.6	0.75
IgA	0.019	0.006	30.6	0.14	0.075	52.2	757.0	325.5	43.0	<0.0001
IgE ^d	0.17	0.17	101.7	1.3	0.33	25.3	2912.3	3792.2	130.2	<0.0001

^a n = 34 for IgM, IgG1, IgG2; n = 33 for IgG3; n = 30 for IgG4; n = 15 for IgA and IgE^b % Recovery refers to the amount of protein from DBS as a percentage of that of plasma value^c unpaired t test results;^d IgE reported as ng/ml

Table 6

Ig isotype levels (mg/dl) from CBDBS versus NDBS

Isotype	CBDBS ^a	NDBS ^b	Ratio ^c	p ^d
IgA	0.14 ± 0.019 ^e	0.17 ± 0.16	0.016	0.54
IgG1	259.3 ± 13.1	309.0 ± 13.2	0.84	0.043
IgG2	77.9 ± 8.7	84.5 ± 5.1	0.92	0.52
IgG3	20.3 ± 3.0	35.6 ± 2.7	0.57	0.0037
IgG4	15.3 ± 3.3	22.8 ± 4.5	0.67	0.37
IgM	3.6 ± 0.43	3.3 ± 0.16	1.1	0.19
IgE	1.3 ± 0.086 ^f	3.5 ± 0.24	0.37	0.0010

^a 34 CBDBSs assayed;^b 108 NDBSs assayed;^c CBDBS/NDBS;^d t test;^e mean ± SEM;^f IgE reported as ng/ml