Journal of Immunological Methods 493 (2021) 113019

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Dried blood spots for the quantitative evaluation of IgG isotypes and correlation with serum samples in HIV-exposed uninfected (HEU) infants

Silvia Baroncelli [a,*], Clementina Maria Galluzzo [a], Giuseppe Liotta [b], Mauro Andreotti [a], Haswell Jere [c], Richard Luhanga [c], Jean Baptiste Sagno [c], Fausto Ciccacci [d], Stefano Orlando [b], Roberta Amici [a], Maria Cristina Marazzi [e], Marina Giuliano [a]

[a] National Center for Global Health, Istituto Superiore di Sanità, Rome, Italy
[b] Department of Biomedicine and Prevention, University of Rome Tor Vergata, Rome, Italy
[c] DREAM Program, Community of S. Egidio, Blantyre, Malawi
[d] Saint Camillus International University of Health Sciences, Rome, Italy
[e] Department of Human Sciences, LUMSA University, Rome, Italy

ARTICLE INFO

Keywords:
IgG subclasses
Dried Blood Spot
Infants

ABSTRACT

Background: The determination of IgG levels and their subclasses can provide clinically relevant information on the status of the immune system. Here we determined the sensitivity and reproducibility of the quantification of IgG subclasses from Dried Blood Spots (DBS) in Malawian uninfected infants exposed to HIV (HEU).

Methods: Sixty paired samples of serum and DBS from HEU infants were used. Samples were collected from 1, 6, and 24-month old infants. IgGs concentrations from both serum and DBS were analyzed by BN ProSpec Siemens assay, using a different setting for sample dilutions. The reproducibility of the DBS method was tested on 10 samples run twice, starting from the DBS extraction process. To assess the systematic, proportional, and random differences, we computed the Passing-Bablok regression, and the Bland-Altman analysis to estimate the total mean bias between the two tests.

Results: The IgG isotypes concentrations from serum and DBS showed significant differences in all the comparisons. Generally, the DBS method underestimated IgG subclasses' values showing a recovery range between 51.2% and 77.6%. Passing Bablok regression on age-based groups showed agreement for IgG, IgG1, and IgG2, but not for IgG3 and IgG4. The mean bias obtained with the Bland Altman test varied largely depending on IgG isotypes (−0.02–2.21 g/l) Coefficient of variation <7.0% was found in the repeated tests for IgG, IgG1, IgG3, and IgG4, while it was 12.4% for IgG2.

Conclusions: Varying degrees of differences were seen in the IgGs measurement in the two different matrices. In IgGs analysis, the DBS method offers promise for population-based research, but the results should be carefully evaluated and considered as a relative value since they are not equivalent to the serum concentrations.

1. Introduction

The use of Dried Blood Spots (DBS) in clinical practice is well established for neonatal screening and in settings where more invasive sample techniques, or procedures as frozen specimen storage and transportation, are problematic.

In the last years, several publications have outlined the utility of DBS for the diagnosis and monitoring of infectious diseases (Lim, 2018; Shimakawa et al., 2021) as well as for large-scale seroprevalence studies (Parker and Cubitt, 1999; Brindle et al., 2014). Despite the potential advantages of DBS as a method of specimen collection, some disadvantages exist, mostly linked to the lack of systematic optimization of extraction procedures, especially for quantitative determinations which require a consistent sample volume to obtain comparable results. Since most of the analyses are performed on serum/plasma, different protocols have been developed trying to improve and to correct the inevitable impact of important factors, such as the size of the DBS punches, the hematocrit effect and the homogeneity of the sample (Hewawasam et al., 2018; Denniff and Spooner, 2010; De Kesel et al., 2014). Moreover, during the phase of matrix recovery, the volume needed for the

* Corresponding author at: National Center for Global Health, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy.
E-mail address: silvia.baroncelli@iss.it (S. Baroncelli).

https://doi.org/10.1016/j.jim.2021.113019
Received 7 September 2020; Received in revised form 24 February 2021; Accepted 4 March 2021
Available online 8 March 2021

correct elution can dilute in excess the metabolite to analyze, producing invalid results. This is particularly true when metabolites in the samples are present in very low concentrations.

In HIV pregnancy the immunoglobulin transplacental passage is impaired (Palmeira et al., 2012; Cumberland et al., 2007), causing an abnormal composition and distributions in IgG and IgG subclasses in neonates (Ray et al., 2019; Baroncelli et al., 2020). In the first months of life IgGs of maternal origin do have a fundamental role in sustaining the humoral responses against pathogens in neonates, and in driving the dynamic process of immunoglobulin development and maturation in infants (Fink et al., 2008). Unbalanced distribution of IgG subclasses (very low levels of IgG2 and unusually high levels of IgG1 and IgG3) has been described in HEU infants and seems to persist up to two years of life, suggesting alterations in IgG maturation (Baroncelli et al., 2020).

The potential use of DBS in the detection of IgG isotypes from DBS in cord blood and neonates has been analyzed by Anderson and colleagues using multiplex Luminex assay; although the IgGs recovery from plasma and DBS was not comparable, the analysis showed reproducible IgGs values within the range of newborn plasma levels (Andersen et al., 2014). The study was conducted on healthy infants, which have a different IgG isotypes profile from that found in HEU infants.

The aim of the present study was to optimize the Dried Blood Spot-based protocol for the determination of IgG subclasses in HEU infants in research projects using a nephelometric method and to determine its performance using serum samples as reference.

2. Materials and methods

2.1. Patients and samples

Dried Blood Spots and serum samples were obtained by infants participating in an observational study conducted in Malawi (SMAC, Safe Milk for African Children, enrollment: 2008–2009), investigating the safety and efficacy of antiretroviral therapy (ART) administration in HIV+ pregnant and lactating women. Study design, clinical details, and antiretroviral strategies have been previously described (Giuliano et al., 2013). Ethics approval was received by the National Health Research Committee, Ministry of Health, Lilongwe, Malawi (approval number #486). Written consent was obtained from all individual participants included in the study before data collection.

Inclusion criteria for this methodologic sub-study were based on the availability of paired DBS and serum samples from HIV exposed uninfected infants (HEU).

2.2. Study design

Sixty infants' paired samples (serum-DBS) were analyzed to determine IgG concentrations and IgG subclasses at 3 time points, for a total of 300 tests (60 determinations of total IgG and 60 for each of the 4 isotypes). Since IgGs concentrations in infants evolve in a significant manner over time, we determined IgGs concentrations at 1, 6, and 24 months of life.

2.3. Sample preparation methods

Serum and DBS were collected from HEU infants attending the Drug Resource Enhancement Against AIDS and Malnutrition (DREAM) Program, managed by the Community of S. Egidio in Blantyre, Malawi. Procedures were performed by locally trained people. After centrifugation at 800–1000 $\times g$ for 15 min of the collected blood, aliquots of serum were stored at −80 °C. For Dried Blood Spot preparation, the plantar surface of infants heel was pricked with sterile lancets and drops of blood were absorbed onto each circle of Whatman 903 filter paper card. Briefly, after the puncture the first blood drop was wiped away, then the filter paper was soaked with a larger drop of blood, until the circle of the spot was completely filled of blood. The procedure was repeated to fill the remaining circles, with successive blood drops. If blood flow was diminished, intermittent gentle pressure was applied to the area surrounding the puncture.

DBS were dried at room temperature for 4 h and then stored in individual ziplock bags containing a desiccant until shipment to our laboratories at the Istituto Superiore di Sanità in Rome, Italy, where the DBS and serum samples were stored at −20 °C and − 80 °C respectively, until processing.

2.4. Serum analysis

Serum was diluted 1:5 with buffer solution and read with the nephelometry. The dilution was needed due to the limited amount of serum available. For each IgG isotype a specific curve, fitting with the serum concentration expected, was selected (Table 1).

2.5. Elution of blood from DBS

Two spots from the filter card were used for each sample. Ten 3.2 mm DBS discs were punched from each spot, using a pneumatic DBS-Dried Blood Spot Card Punch (Analytical Sales and Services Inc.,Flanders, NJ). The final 20 punches derived from the two spots were placed together into a low binding flat-bottom 24-well plate covered with a lid and incubated overnight at + 4 °C in 400 μl of elution buffer, Phosphate Buffered Saline (PBS 1× Sigma Aldrich, Milan, Italy) + 0.05% Tween 20 (Sigma Aldrich, Milan, Aldrich) + 0.1% BSA (Sigma Aldrich, Milan, Italy) gently shaken with a bench-top shaker. For elution, we used a methodology already described (Mercader et al., 2006). Briefly, after incubation the soaked punches and elution buffer were transferred into the corresponding centrifuging system, consisting of a 15 mL centrifuge tube (Falcon Polypropylene Conical Tubes, Corning Science) that held a microtube (1.2 ml Corning Cluster Tubes, Salt Lake City, UT), and supported an uncapped 2.5 mL syringe barrel at the open end. Samples were centrifuged at room temperature for 7 min 1800 RPM at RT. Eluate (median recovered volume: 330 μl) was transferred in 1.5 low-binding vials (Protein LoBind Tube, Eppendorf) and centrifuged (14,000 RPM, 15 min RT) to remove debris.

Based on previous literature (Andersen et al., 2014), a 3.2 mm punch was considered to contain 3.275 μl of blood; considering a hematocrit value of 50% as acceptable for infants (Jopling et al., 2009; Hall et al., 2015), we considered 1.6375 μl of plasma for each 3.2 mm punch reaching a final dilution of 1:12.2 (32.75 μl (1.6375 μl × 20 spots) in 400 μl of elution buffer).

2.6. Quantification of IgG and subclasses

Total IgG and IgG subclass levels in serum and DBS samples were determined using IgG total, IgG1, IgG2, IgG3, and IgG4 reagents (Siemens, Siemens Healthcare Diagnostics) and read by an automatized nephelometry (BN ProSpec® System analyzer, Siemens Healthcare Diagnostics).

2.7. Reproducibility of the assay

Ten DBS samples from 10 individuals were tested twice starting from the elution process, and the coefficient of variation (CV) was used to interpret the consistency of the results.

2.8. Statistical analysis

Analysis of the data was performed using SPSS V26 software (IBM Corp, Armonk, NY, USA). Normal distribution was checked using the Shapiro-Wilk test, which revealed a non-normal distribution of data. Values are expressed in medians and interquartile range. The percentage of recovery was used to determine the difference between the results obtained from serum and those obtained from DBS. The Wilcoxon test

Table 1
Percentage of quantifiable serum and DBS samples using BNI II Siemens nephelometry assay. The calibration curve ranges are also reported. Serum samples were pre-diluted 1:5, and DBS elution were pre-diluted 1:12.2.

	Serum			DBS			*Calibration curve*
	Samples pre-dilution 1:5			*Samples pre-dilution 1:12.2*			
	Sample (n)	*Valid (%)*	*dilution*	*Sample (n)*	*Valid (%)*	*dilution*	*Range (g/l)*
Total IgG	*60*	*100*	*1:400*	*60*	*100*	*1:100*	*0.07–46.0*
IgG1	*60*	*100*	*1:100*	*60*	*100*	*1:20*	*0.04–27.0*
IgG2	*60*	*100*	*1: 20*	*39*	*65.0*	*1:1**	*0.09–11.0*
IgG3	*60*	*100*	*1:2000*	*60*	*100*	*1: 100*	*0.0017–2.1*
IgG4	*60*	*100*	*1:400*	*32*	*53.3*	*1: 100*	*0.0026–3.3*

*To determine the IgG2 concentration in DBS samples the nephelometric software was manually modified to reach the detection limit of IgG2 to 0.00174 g/l.

was used to determine the statistical difference between the paired samples.

Due to the non-parametric conditions, the inter-methods correlation was calculated with the Spearman test and the Passing-Bablok regression (Bilić-Zulle, 2011), using XSTAT software (Statistical Software for Excel, Microsoft Inc., Seattle). In this regression technique the intercept (representing constant bias) and the slope (representing proportional bias) are presented as estimates and should ideally not be different from 0 (intercept) and 1 (slope), at $p < 0.05$ (Passing and Bablok, 1984). Bland Altman analysis for relative differences was also performed to visualize the correlation between bias and concentrations (Bland and Altman, 1986).

3. Results

A total of 300 tests from serum and 300 from DBS were performed, to obtain the IgG subclasses profile of 60 infants in three different groups of age.

3.1. Sample matrix effects on nephelometric assay sensibility

Valid determinations were obtained for all serum samples analyzed with the nephelometric method, using different settings to obtain an optimal range of concentrations for each IgG subclasses (Table 1). Similarly, the eluted solution from DBS (pre-diluted 1:12.2) always generated quantifiable results for total IgG, IgG1, and IgG3, independently from the age-group. On the other hand, quantifiable results for IgG2 and IgG4 were obtained only for 65%, and 53%, respectively, of the DBS analyzed (Table 1). Most of the DBS samples with non-quantifiable levels of IgG2 (<0.0174 g/l) belonged to 1 month-old infants, which had corresponding serum IgG2 levels ranging from 0.145 g/l to 0.868 g/l (median: 0.52 g/l, IQR: 0.45, and 0.59). Similarly, the 53% of IgG4 determinations from DBS were not quantifiable when the corresponding serum samples ranged from 0.001 g/l to 0.068 g/l (median: 0.0275 g/l, IQR: 0.0014–0.0041).

3.2. Comparative analysis of IgGs in serum and DBS

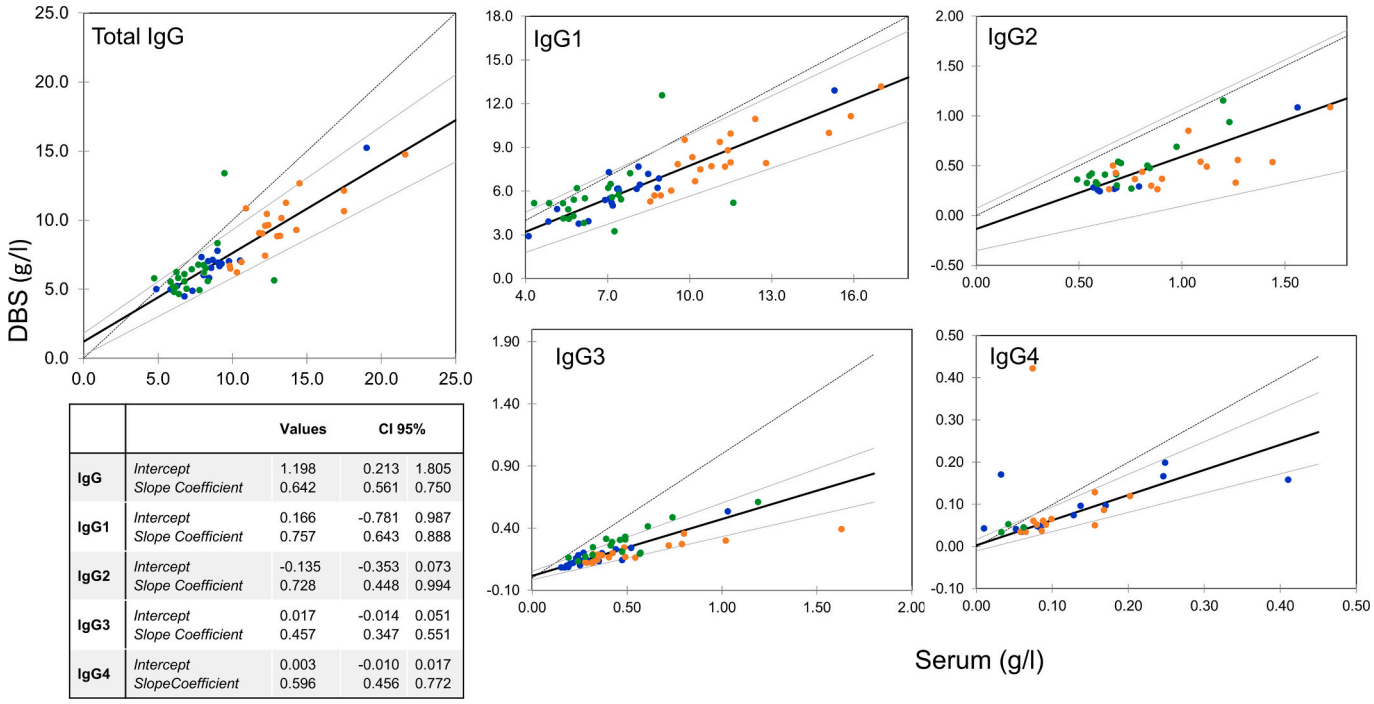
The IgGs concentrations obtained from paired serum/DBS samples are reported in Table 2. IgGs levels in all subclasses showed significant differences when analyzed in serum and DBS ($p < 0.001$) Generally, the IgGs levels determined from the DBS matrix were lower than those obtained from serum. The percentage of recovery from the DBS samples was 77.0% (IQR: 68.4–86.3) for total IgG and 77.6% (IQR: 69.8–87.9) for IgG1 of the serum values, respectively. A low recovery from DBS was even more evident for IgG2, IgG3, and IgG4 (percentage of recovery of 54.2% (IQR: 40.9–70.7) 51.4% (IQR: 41.5–59.6), and 63.0% (57.6–80.6), respectively).

There were statistically significant correlations between DBS and serum concentrations by Spearman analysis for all the IgG subclasses, ranging from $r = 0.593$ to 0.846 ($p < 0.0001$), but the Passing-Bablok analysis revealed a poor agreement between the two methods (Fig. 1).

Table 2
IgGs concentrations (g/l) of serum and DBS paired samples. Values are expressed as medians and IQR. Recovery indicates the percentage of IgGs concentration recovered from DBS samples compared to serum concentration.

	n	serum (g/l)	n	DBS (g/l)	Recovery (%)
total IgG					
month 1	20	8.48 (7.44–9.13)	20	6.77 (5.39–7.10)	*77.0 (72.5–84.3)*
month 6	20	6.84 (6.19–8.19)	20	5.73 (5.14–6.40)	*84.8 (73.8–92.5)*
month 24	20	12.35 (11.3–14.13)	20	9.46 (7.78–10.81)	*76.0 (67.5–83.3)*
overall	60	8.89 (7.00–12.15)	60	6.77 (5.70–9.01	*68.9 (66.0–78.6)*
IgG1					
month 1	20	7.27 (6.45–8.16)	20	6.1 (4.84–6.78)	*78.5 (71.5–84.4)*
month 6	20	6.15 (5.57–7.37)	20	5.30 (4.41–6.08)	*87.4 (73.6–95.7)*
month 24	20	10.95 (9.63–12.18)	20	7.94 (6.89–9.84)	*70.8 (65.5–82.3)*
overall	60	7.66 (6.20–10.18)	60	6.17 (5.18–7.71)	*77.6 (69.8–87.9)*
IgG2					
month 1	7	0.67 (0.59–0.79)	7	0.28 (0.26–0.29)	*41.4 (40.1–50.2)*
month 6	17	0.68 (0.57–0.84)	17	0.42 (0.33–0.53)	*65.6 (57.1–74.7)*
month 24	15	0.90 (0.77–1.26)	15	0.44 (0.33–0.54)	*44.0 (37.1–63.3)*
overall	39	0.75 (0.62–1.03)	39	0.41 (0.30–0.54)	*54.2 (40.9–70.7)*
IgG3					
month 1	20	0.25 (0.19–0.35)	20	0.14 (0.10–0.19)	*52.8 (50.4–56.6)*
month 6	20	0.44 (0.32–0.55)	20	0.23 (0.18–0.31)	*63.1 (51.9–68.5)*
month 24	20	0.39 (0.32–0.68)	20	0.17 (0.13–0.26)	*40.8 (34.9–46.4)*
overall	60	0.35 (0.27–0.49)	60	0.18 (0.13–0.26)	*51.2 (41.5–59.6)*
IgG4					
month 1	15	0.08 (0.05–0.17)	15	0.05 (0.43–0.16)	*61.0 (58.1–80.2)*
month 6	3	0.042 (0.03 -)	3	0.05 (0.03 -)	*103.0 (72.4 -)*
month 24	14	0.09 (0.07–0.16)	14	0.06 (0.04–0.10)	*63.3 (54.2–72.7)*
overall	32	0.082 (0.06–0.15)	32	0.052 (0.04–0.10)	*63.0 (58.0–80.6)*

The hypothesis of similarity was rejected (both slopes and intercepts did not include the target values of 1 and 0 respectively), and the equation created for the assessment of the agreement between the 2 methods showed a constant bias ranging for the different IgG subclasses from −0.135 to 1.198 and a proportional bias ranging from 0.457 to 7.28. To overcome the large age-related variability of the data, the regression



		Values	CI 95%	
IgG	*Intercept*	1.198	0.213	1.805
	Slope Coefficient	0.642	0.561	0.750
IgG1	*Intercept*	0.166	-0.781	0.987
	Slope Coefficient	0.757	0.643	0.888
IgG2	*Intercept*	-0.135	-0.353	0.073
	Slope Coefficient	0.728	0.448	0.994
IgG3	*Intercept*	0.017	-0.014	0.051
	Slope Coefficient	0.457	0.347	0.551
IgG4	*Intercept*	0.003	-0.010	0.017
	SlopeCoefficient	0.596	0.456	0.772

Fig. 1. Comparison of IgGs levels in DBS and plasma by Passing Bablok regression analysis ($n = 60$). with the regression line (solid line), the CI for the regression line (grey lines), and identity line (x = y, dotted line). The different dot colors indicate the different age of the infant's samples. 1 month: blue; 6 months: green; 24 months: orange. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

analysis was filtered for the age-groups ($n = 20$) (Table 3). A better agreement between the two methods can be seen; the values obtained for IgG, IgG1, and IgG2 with DBS and serum were comparable within the investigated concentration range (including 0 in intercept and 1 in slope), while systemic and/or proportional differences between matched samples were found in IgG3 and IgG4 analysis.

The Bland Altman plots quantified the mean bias between the two methods, which ranged from −2.8 g/l for IgG to −0.02 g/l for IgG4 (Fig. 2).

3.3. *Reproducibility of IgGs analysis from samples of the same individual extracted from different DBS punches*

To assess the reproducibility of the extraction method, we processed 2 series of punches from DBS of the same individual. Ten filter cards, each containing four spots from the same individuals, were used to obtain two different eluates, which were analyzed to quantify IgGs concentrations. The mean coefficient of variation of total IgG was 6.14% (CI: 0.20–1.30). IgGs subclasses showed coefficient of variation below 6%, and acceptable interval of confidence; IgG1, (6.14%, CI: 0.20–1.30), IgG3 (5.6%, CI: 3.2–8.7) and IgG4 (5.9%, CI: −0.1–12.0). For IgG2 only 6 samples in test I and 7 samples in test II were quantifiable; the mean coefficient of variation was 12.4%, with a large confidence interval (−2.8–22.9), indicating a significant discrepancy in repeated measurements.

4. Discussion

Here we report the results of the quantification of IgG subclasses analyzed on different matrices, serum, and DBS. The results, based on

Table 3
Results of Passing Bablok regression analysis on age-based groups.

	Age (months)	*Systemic differences*		*Proportional differences*		*Random differences*	
		Intercept	*95% CI*	*Slope*	*95% CI*	*RSD*	*interval + 1.96 RSD*
IgG	1	0.676	−0.107 2.722	0.693*	0.411 0.888	0.584	±1.445
	6	0.667	−5.327 4.121	0.739	0.253 1.616	1.519	±2.976
	24	−0.843	−6.934 2.407	0.758	0.555 1.323	1.134	± 2.222
IgG1	1	−1.381*	−4.969 -0.224	0.977	0.815 1.477	0.533	±1.045
	6	−0.629	−8.72 2.986	0.972	0.387 2.276	1.429	±2.801
	24	−0.899	−5.917 1.601	1.379	0.999 2.201	0.708	±1.388
IgG2	1	−0.014	−0.670 0.214	0.475	0.100 1.400	0.159	+0.311
	6	−0.177*	0.138 0.003	0.906	0.607 1.326	0.083	±1.635
	24	−0.093	−0.614 0.298	0.554	0.174 1.074	0.155	+0.303
IgG3	1	0.003	−0.014 0.019	0.519**	0.453 0.583	0.018	±0.039
	6	0.012	−0.054 0.090	0.604**	0.421 0.767	0.067	±0.131
	24	0.039	−0.019 0.008	0.298**	0.206 0.620	0.044	±0.087
IgG4	1	0.004	−0.010 0.025	0.563**	0.336 0.742	0.044	±0.086
	6	–	–	–	–	–	–
	24	−0.020	−0.178 0.015	0.861**	0.461 2.500	0.089	±0.174

* Intercepts CI does not contain the value 0; the two methods might differ by at least by a constant amount.
** Slopes CI does not contain the value 1; there could be a proportional difference between the two methods.

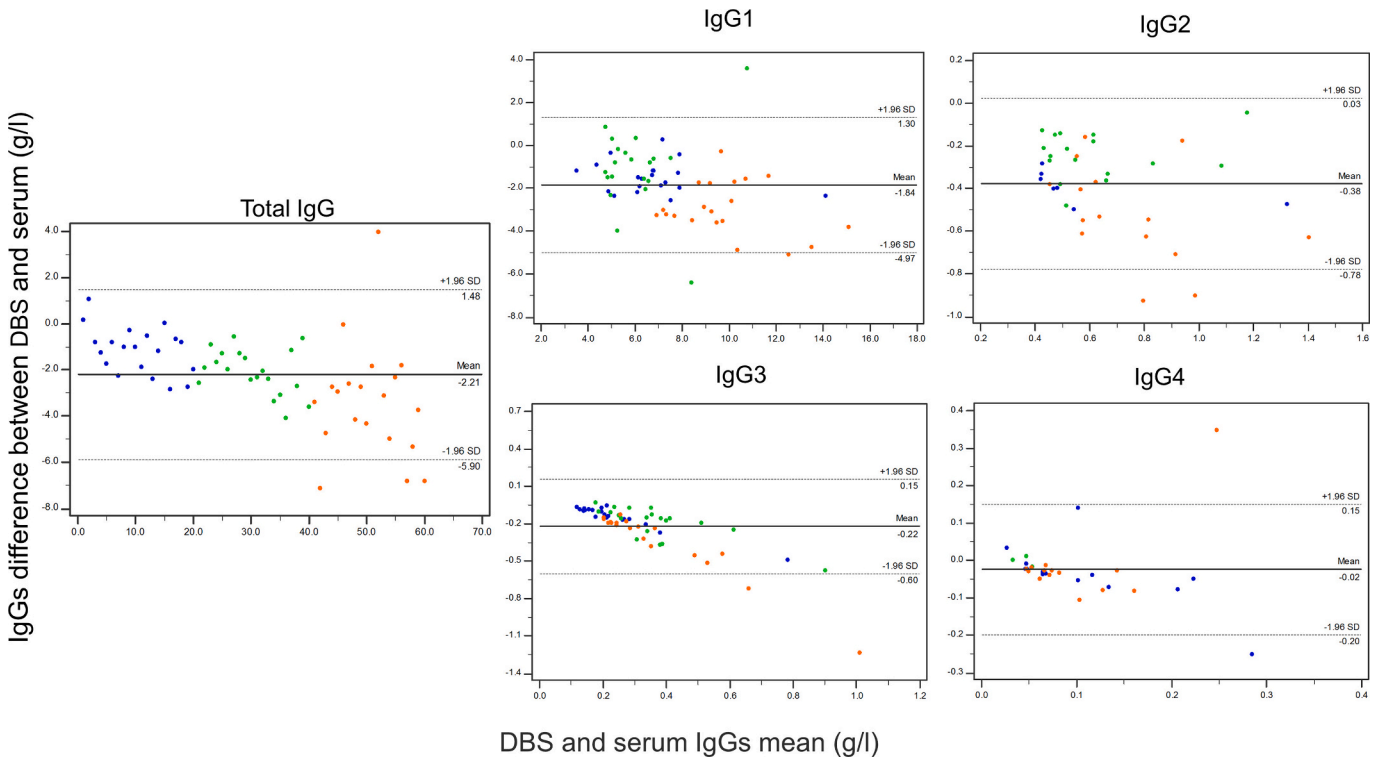


Fig. 2. Bland–Altman plots showing the difference between IgG isotype concentration in DBS and serum. The solid line represents the mean of differences (bias) and dotted lines the limits of agreement (LOA). The different dot colors indicate the different age of the infant's samples. 1 month: blue; 6 months: green; 24 months: orange. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

paired samples reveal that the DBS method underestimates the value obtained with the serum to an extent of 30–50%. The statistical tools that we employed were consistent in showing that the two methods can not be used interchangeably; importantly, when IgG evaluations of paired samples were compared within age-based groups, most of the results fell within required criteria of regression, indicating a constant and proportional bias between the two sets of data (represented by intercept and slope within 0 and 1). Our results confirm the predictable differences between the two quantitative methods and provide specific information to the investigators regarding how to design and carefully interpret the quantification of immunoglobulin levels when analyzed on the DBS matrix.

The determination of IgG levels and their subclasses can provide information on the status of the immune system (Crum-Cianflone et al., 2012); the HIV infection alters the transplacental passage of maternal IgG, impacting the immunological protection of the infants during the first months of life. Since 2000, the use of antiretroviral therapy during pregnancy has reduced globally the case of new pediatric HIV infections by nearly 75% (United Nations Children's Fund, For Every Child, End AIDS, 2016), increasing the number of HIV exposed uninfected (HEU) infants, which in some African regions can account up to 30% of births (Wirth et al., 2020). This fragile population shows a high rate of vulnerability to the infections (Slogrove et al., 2020); it is believed that the immunological dysfunction in HEU infants might be linked to an *in utero* impact of HIV and of antiretroviral therapy which can alter the development of the fetal immune system (Brennan et al., 2016; Ruck et al., 2016). The IgGs distribution in infants reflects the maternal immunological alteration; the HIV-related hypergammaglobulinemia in mothers has a relevant impact on IgGs transplacental passage, causing in infants important alterations in IgGs distribution and maturation (Baroncelli et al., 2019).

We selected samples from HEU infants of different ages since the development of each IgG subclass during the first two years of life has a different age of onset and speed of synthesis, and this heterogenicity can indicate abnormalities in the process of immune maturation. In this view, it is important to standardize the use of DBS, which has become the preferred non-invasive blood sampling in remote or under-resourced geographical regions, where environmental conditions can preclude the appropriate cold chain of transportation and storage of biological samples (Calafat and Kato, 2014; Freeman et al., 2018). In our study the DBS hemolysis did not seem to interfere with the automatized BN ProSpec® System protein analyzer; the erythrocytes (RBC) lysis can not be prevented in the DBS method since the breakage of the erythrocytes is an un-evitable part of the elution process; based on colorimetric confrontation we estimated that the final product of elution was containing more than 1000 mg of Hb per ml (Centers for Disease Control and Prevention, Quick-Reference Tool for Hemolysis Status, 2019). Although we can not completely exclude some degree of interference, we are quite confident that the hemolysis in our samples was not the principal cause of unquantifiable or underestimation in sample readings; the automatic system did not show any automatic alarms (provided by the software system) and the results for all isotypes (obtained from the same tube), showed different degree of underestimation even when they were read at the same internal dilution (*i.e.* 1:100 for IgG3 and IgG4). Indeed, the un-quantifiable DBS samples corresponded to the sera expressing very low IgGs titers. This occurred, to a large extent, in the determination of IgG2 and IgG4 levels in 1, 6, and 24 months old infants. We can only speculate that conformational differences in isotypes structures, such as hinge conformation, number of disulfide bonds, susceptibility to the proteolytic enzymes (Abramov et al., 1983; Vidarsson et al., 2014), might have had a role interfering in the process of epitopes binding necessary for detection. The better percentage of IgGs recovery from the DBS matrix occurred in six months old infants, in correspondence to the onset of IgG synthesis in infants and the gradual decline of circulation of maternal IgG. Although the reason is not clear, we can speculate that the IgGs titers in 6-month-old infants could correspond to an adequate concentration for an optimal DBS extraction. In this view, the DBS methodology has to face the delicate balance

between obtaining a sufficient volume for a complete elution process and an adequate dilution for the metabolite analyses. In our experience, we found a good equilibrium with 20 punches (3.2 mm) eluted in a volume of 400 µl dilution buffer.

Many attempts were performed with different elution volume or higher number of spots, but the decreasing elution volumes resulted in an incomplete IgGs recovery, and a larger number of spots required an increased volume of elution that did not improve the IgGs recovery.

The Passing-Bablok regression analysis revealed a significant linear relationship (Spearman test, $p < 0.001$) but not their agreement; nor intercept nor slope included 0 and 1, indicating poor agreement between the two sets of data. Considering that the widespread sample distribution, with significantly different age-related IgGs titers (Baroncelli et al., 2020) might have an impact on the regression results, we repeated the analysis for groups of age. The new test revealed that total IgG (at 6 and 24 months), and IgG1 and IgG2 (in all age groups) respected the criteria of regression, indicating no proportional differences (slope CI 95% including 1), nor systematic difference (intercept CI 95% including 0) between the two methods. On the other hand, IgG3 and IgG4 values displayed systemic differences and discordance between the two tests. The interpretation of data seems to indicate that the discrepancy between the DBS and serum values is not constant across the whole range of measurements but could be different at low levels as opposed to high levels. This can also be visualized in the Bland-Altman plots, which show a different sample distribution around the mean bias related to age. The results reflected the underestimation already observed, which have a different degree of discrepancy through the IgG classes. However it is important to note that the same longitudinal IgGs trend was observed over time for both methods, indicating that the DBS method can be reliably used in the context of research studies.

In the current study, the reproducibility of the assays showed reasonable results; the coefficient of variation that we found in the repeated analysis was consistently within 7% for all IgG isotypes and only IgG2 showed a higher variability, which seems to reflect the IgG2 determination near the assay quantification limits. Considering that the analysis was repeated on samples from the same individual from a different set of punches, we can be confident to have a good standardization of the elution process, during which different factors can influence the final results.

This study has limitations, some of them intrinsic to the DBS system; first, the hematocrit effect, which directly impacts on plasma/blood ratio and can limit the homogeneity of spotting on filter paper (Freeman et al., 2018). Moreover, capillary samplings in neonates generally show higher hematocrit values than venous samples (Esan, 2016) enhancing the discrepancy in DBS/serum comparison. The long term storage of our samples (more than 10 years) is another limit in the study. In previous studies, the detection of nucleic acids from DBS was still reliable after 5–15 years of cool storage (Malsagova et al., 2020), but only a few data are available on long term stability of proteins or other metabolites; although IgGs are considered relatively stable proteins, and the samples in this study had received appropriate storage (−80 °C for serum and −20 °C for DBS) we cannot exclude a time differential impact on IgG isotypes stability. Another limitation of the study is that the volume necessary to elute the spots brought the dilutions of the samples near the assay limit for nephelometric assays. Nevertheless, the nephelometric system has the important advantage in producing highly reproducible results, even when working in the lower portion of the calibration curves.

5. Conclusions

In conclusion, the DBS system in the quantification of IgGs should be used carefully: the levels obtained using DBS are generally lower than the corresponding serum levels and the statistical analysis, although showing a significant correlation between the two determinations, underlined a discrepancy between the two methods We believe therefore that the DBS method for its practical advantages in the collection, storage and shipment can be reliably used in the context of research studies, while its use in the diagnostics laboratories could be limited.

Declaration of Competing Interest

None.

Acknowledgments

This study was supported by a grant from the Italian Agency for Cooperation and Development (Grant n. AID:01111/03/04) and a grant from the Ministry of Health, Rome, Italy (grant no. 3C04/1). The authors have no conflict of interest to declare. We thank Alessandra Mattei and Stefania Donnini for providing secretarial help, and Ferdinando Costa and Patrizia Cocco, for technical support. We are grateful to Riccardo d'Amico for the precious technical support with BN ProSpec® System Analyzer.

References

Centers for Disease Control and Prevention, 2019. Quick-reference tool for hemolysis status. In: Hemolysis Reference Palette. Available at: https://www.cdc.gov/ncezid/dvbd/pdf/Hemolysis_Palette_Bookmark-P.pdf (Accessed: June 2020).

Abramov, V.M., Arkhangelskaya, Z.A., Zav'yalov, V.P., 1983. Conformational properties of human immunoglobulin G subclasses. Analysis by temperature-perturbation and solvent-perturbation spectroscopy. Biochim. Biophys. Acta 742, 295–302. https://doi.org/10.1016/0167.

Andersen, N.J., Mondal, T.K., Preissler, M.T., Freed, B.M., Stockinger, S., Bell, E., Druschel, C., Louis, G.M., Lawrence, D.A.J., 2014. Detection of immunoglobulin isotypes from dried blood spots. Immunol Methods 404, 24–32. https://doi.org/10.1016/j.jim.2013.12.001.

Baroncelli, S., Galluzzo, C.M., Liotta, G., Orlando, S., Ciccacci, F., Andreotti, M., Mpwhere, R., Luhanga, R., Sagno, J.B., Amici, R., Marazzi, M.C., Giuliano, M., 2019. IgG abnormalities in HIV-positive Malawian women initiating antiretroviral therapy during pregnancy persist after 24 months of treatment. Int. J. Infect. Dis. 88, 1–7. https://doi.org/10.1016/j.ijid.2019.09.001.

Baroncelli, S., Galluzzo, C.M., Liotta, G., Andreotti, M., Orlando, S., Ciccacci, F., Jere, H., Luhanga, R., Sagno, J.B., Amici, R., Marazzi, M.C., Giuliano, M., 2020. Dynamics of immunoglobulin G subclasses during the first two years of life in Malawian infants born to HIV-positive mothers BMC. Pediatr 20, 181. https://doi.org/10.1186/s12887-020-02091-z.

Bilić-Zulle, L., 2011. Comparison of methods: Passing and Bablok regression. Biochemia Medica 21, 49–52.

Bland, J.M., Altman, D.G., 1986. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 327, 307–310.

Brennan, A.T., Bonawitz, R., Gill, C.J., Thea, D.M., Kleinman, M., Useem, J., Garrison, L., Ceccarelli, R., Udokwu, C., Long, L., Fox, M.P., 2016. A meta-analysis assessing all-cause mortality in HIV-exposed uninfected compared with HIV-unexposed uninfected infants and children. AIDS 30, 2351–2360. https://doi.org/10.1097/QAI.0000000000002097.

Brindle, E., O'Connor, K.A., Garrett, D.A., 2014. Applications of dried blood spots in general human health studies. In: Dried Blood Spots: Applications and Techniques. John Wiley & Sons, Inc, Hoboken (NJ), pp. 114–129.

Calafat, A.M., Kato, K., 2014. Applications of dried blood spots in environmental population studies. In: Dried Blood Spots: Applications and Techniques. John Wiley & Sons, Inc, Hoboken (NJ), pp. 130–139.

Crum-Cianflone, N.F., Collins, G., Defang, G., Iverson, E., Eberly, L.E., Duplessis, C., Maguire, J., Ganesan, A., Agan, B.K., Lalani, T., Whitman, T., Brandt, C., Faix, D., Blair, P.J., Burgess, T., 2012. Immunoglobulin G subclass levels and antibody responses to the 2009 influenza A (H1N1) monovalent vaccine among human immunodeficiency virus (HIV)-infected and HIV-uninfected adults. Clin Exp Immunol 168, 135–141. https://doi.org/10.1111/j.1365-2249.2011.04550.x.

Cumberland, P., Shulman, C.E., Maple, P.A., Bulmer, J.N., Dorman, E.K., Kawuondo, K., Marsh, K., Cutts, F.T., 2007. Maternal HIV infection and placental malaria reduce transplacental antibody transfer and tetanus antibody levels in newborns in Kenya. J. Infect. Dis. 196, 550–557. https://doi.org/10.1086/519845.

De Kesel, P.M.M., Capiau, S., Lambert, W.E., Stove, C.P., 2014. Current strategies for coping with the hematocrit problem in dried blood spot analysis. Bioanalysis 6, 1871–1874. https://doi.org/10.4155/bio.14.151.

Denniff, P., Spooner, N., 2010. The effect of hematocrit on assay bias when using DBS samples for the quantitative bioanalysis of drugs. Bioanalysis 2, 1385–1395. https://doi.org/10.4155/bio.10.103.

Esan, A.J., 2016. Hematological differences in newborn and aging: a review study. Hematol Transfus Int J 3, 178–190. https://doi.org/10.15406/htij.2016.03.00067.

Fink, K., Zellweger, R., Weber, J., Manjarrez-Orduno, N., Holdener, M., Senn, B.M., Hengartner, H., Zinkernagel, R.M., Macpherson, A.J., 2008. Long-term maternal

imprinting of the specific B cell repertoire by maternal antibodies. Eur. J. Immunol. 38, 90–101. https://doi.org/10.1002/eji.200737872.

Giuliano, M., Andreotti, M., Liotta, G., Jere, H., Sagno, J.B., Maulidi, M., Mancinelli, S., Buonomo, E., Scarcella, P., Pirillo, M.F., Amici, R., Ceffa, S., Vella, S., Palombi, L., Marazzi, M.C., 2013. Maternal antiretroviral therapy for the prevention of mother-to-child transmission of HIV in Malawi: maternal and infant outcomes two years after delivery. PLoS One 8, e68950. https://doi.org/10.1371/journal.pone.0068950.

Hall, E.M., Flores, S.R., De Jesús, V.R., 2015. Influence of hematocrit and total-spot volume on performance characteristics of dried blood spots for newborn screening. Int J Neonatal Screen 1, 69–78. https://doi.org/10.3390/ijns1020069.

Hewawasam, E., Liu, G., Jeffery, D.W., Gibson, R.A., Muhlhausler, B.S., 2018. Estimation of the volume of blood in a small disc punched from a dried blood spot card. Eur. J. Lipid Sci. Technol. 120, 1700362. https://doi.org/10.1002/ejlt.201700362.

Jopling, J., Henry, E., Wiedmeier, S.E., Christensen, R.D., 2009. Reference ranges for hematocrit and blood hemoglobin concentration during the neonatal period: data from a multihospital health care system. Pediatrics 123, e333–e337. https://doi.org/10.1542/peds.2008-2654.

Lim, M.D., 2018. Dried blood spots for global health diagnostics and surveillance: opportunities and challenges. Am J Trop Med Hyg 99, 256–265. https://doi.org/10.4269/ajtmh.17-0889.

Malsagova, K., Kopylov, A., Stepanov, A., Butkova, T., Izotov, A., Kaysheva, A., 2020. Dried blood spot in laboratory: directions and prospects. Diagnostics 10, 248. https://doi.org/10.3390/diagnostics10040248.

Mercader, S., Featherstone, D., Bellini, W.J., 2006. Comparison of available methods to elute serum from dried blood spot samples for measles serology. J Vir Methods 137, 140–149. https://doi.org/10.1016/j.jviromet.2006.06.018.

Palmeira, P., Quinello, C., Silveira-Lessa, A.L., Zago, C.A., Carneiro-Sampaio, M., 2012. IgG placental transfer in healthy and pathological pregnancies. Clin Dev Immunol 985646. https://doi.org/10.1155/2012/985646.

Parker, S.P., Cubitt, W.D., 1999. The use of the dried blood spot sample in epidemiological studies. J. Clin. Pathol. 52, 633. https://doi.org/10.1136/jcp.52.9.633.

Passing, H., Bablok, W., 1984. Comparison of several regression procedures for method comparison studies and determination of sample sizes. Application of linear regression procedures for method comparison studies in Clinical Chemistry, Part II. Clin. Chem. Lab. Med. 22, 431–445. https://doi.org/10.1515/cclm.1984.22.6.431.

Ray, J.E., Dobbs, K.R., Ogolla, S.O., Daud, I.I., Vulule, J., Sumba, P.O., Rochford, R., Dent, A.E., 2019. Reduced Transplacental transfer of antimalarial antibodies in Kenyan HIV-exposed uninfected infants. Open Forum Infect Dis 6, ofz237. https://doi.org/10.1093/ofid/ofz237.

Freeman, J.D., Rosman, L.M., Ratcliff, J.D., Strickland, P.T., Graham, D.R., Silbergeld, E. K., 2018. State of the science in dried blood spots. Clin. Chem. 64, 656–679.

Ruck, C., Reikie, B.A., Marchant, A., Kollmann, T.R., Kakkar, F., 2016. Linking susceptibility to infectious diseases to immune system abnormalities among HIV-exposed uninfected infants. Front. Immunol. 7, 310. https://doi.org/10.3389/fimmu.2016.00310.

Shimakawa, Y., Vernoux, L., Gabassi, A., Mercier-Delarue, S., Vincent, J.P., Simon, F., Maylin, S., 2021. Analytical validation of hepatitis B core-related antigen (HBcrAg) using dried blood spots (DBS). J Viral Hepat. https://doi.org/10.1111/jvh.13489. Online ahead of print.

Slogrove, A.L., Powis, K.M., Johnson, L.F., Stover, J., Mahy, M., 2020. Estimates of the global population of children who are HIV-exposed and uninfected, 2000–18: a modelling study. Lancet Glob. Health 8, e67–e75. https://doi.org/10.1016/S2214-109X(19)30448-6.

United Nations Children''s Fund, 2016. For Every Child, End AIDS – Seventh Stocktaking Report. UNICEF, New York. Available at: https://data.unicef.org/resources/every-child-end-aids-seventh-stocktaking-report-2016/ (Accessed: July 2020).

Vidarsson, G., Dekkers, G., Rispens, T., 2014. IgG subclasses and allotypes: from structure to effector functions. Front. Immunol. 5, 520. https://doi.org/10.3389/fimmu.2014.00520.

Wirth, K.E., Gaolathe, T., Pretorius, Holme M., Mmalane, M., Kadima, E., Chakalisa, U., Manyake, K., Matildah, Mbikiwa A., Simon, S.V., Letlhogile, R., Mukokomani, K., van Widenfelt, E., Moyo, S., Bennett, K., Leidner, J., Powis, K.M., Lebelonyane, R., Alwano, M.G., Jarvis, J., Dryden-Peterson, S.L., Kgathi, C., Moore, J., Bachanas, P., Raizes, E., Abrams, W., Block, L., Sento, B., Novitsky, V., El-Halabi, S., Marukutira, T., Mills, L.A., Sexton, C., Pals, S., Shapiro, R.L., Wang, R., Lei, Q., DeGruttola, V., Makhema, J., Essex, M., Lockman, S., Tchetgen Tchetgen, E.J., 2020. Population uptake of HIV testing, treatment, viral suppression, and male circumcision following a community-based intervention in Botswana (Ya Tsie/BCPP): a cluster-randomised trial. Lancet HIV 7, e422–e433. https://doi.org/10.1016/S2352-3018(20)30103-X.