

Dried Blood Spot Samples for Seroepidemiology of Infections with Human Papillomaviruses, *Helicobacter pylori*, Hepatitis C Virus, and JC Virus

Tim Waterboer¹, Bolormaa Dondog^{1,2}, Kristina M. Michael¹, Angelika Michel¹, Markus Schmitt¹, Salvatore Vaccarella², Silvia Franceschi², Gary Clifford², and Michael Pawlita¹

Abstract

Background: To establish antibody analysis from dried blood spots (DBS) on filter paper for seroepidemiologic infection and cancer association studies, we analyzed data from a population-based study in Mongolia.

Methods: Using multiplex serology, we analyzed 985 paired DBS and serum samples from the same donors for antibodies to 12 different proteins from four groups of infectious agents: human papillomaviruses (HPV), *Helicobacter pylori* (*H. pylori*), hepatitis C virus (HCV), and JC polyomavirus (JCV).

Results: Quantitative antibody reactivities in serum and DBS showed good correlation, with median correlation coefficients (Pearson R^2) of 0.88 (range, 0.80–0.90) for high-titer (i.e., *H. pylori*, HCV, JCV) and 0.79 (range, 0.72–0.85) for low-titer antibodies (i.e., HPV). For high-titer antibodies, serum and DBS data were comparable (median slope of linear trend line, 1.14; range, 1.09–1.21), whereas for low-titer antibodies, DBS reactivities were lower than in serum (median slope, 0.54; range, 0.50–0.80). By extrapolating seropositivity cutoff points previously defined for serum to DBS, we found high agreement (>89% for all antigens) of dichotomized DBS and serum results and median kappa values for high- and low-titer antibodies of 0.86 and 0.78 (range, 0.78–0.92 and 0.55–0.86), respectively. Epidemiologic associations with known risk factors for HPV antibodies were as strong for DBS as for serum.

Conclusions: DBS provide a reliable alternative to serum or plasma for detection of antibodies against various pathogens by multiplex serology.

Impact: DBS do not require blood centrifugation and allow storage and shipment at ambient temperature, thus facilitating field work for seroepidemiologic studies especially in environments with limited technical infrastructure. *Cancer Epidemiol Biomarkers Prev*; 21(2); 287–93. ©2011 AACR.

Introduction

Seroepidemiologic studies of infectious diseases rely on blood-derived samples to detect specific antibodies against infectious agents. In contrast to tissue samples, full blood can be relatively easily obtained. However, centrifugation to separate blood cells from serum or plasma, and sample storage and shipment below 0°C

require technical equipment sometimes not available in low-resource countries or remote field work locations. Therefore, we explored the feasibility of seroepidemiologic studies on the basis of dried blood spots (DBS) on filter paper cards which are commonly used for newborn screening. DBS cards have been used in seroepidemiologic studies of different viral or bacterial infections (1). To allow for a direct comparison, we analyzed paired DBS and serum samples from the same donors derived from intravenous blood of 985 women from a population-based study conducted in Mongolia (2).

Using multiplex serology, a fluorescent bead-based high-throughput method that allows the analysis of serum or plasma samples for antibodies to up to 100 different antigens simultaneously (3), these 1,970 samples were tested for antibodies to 12 different proteins from 4 groups of infectious agents, most of which to our knowledge have not been analyzed by DBS serology: human papillomaviruses (HPV), *Helicobacter pylori* (*H. pylori*), hepatitis C virus (HCV), and JC polyomavirus (JCV).

Authors' Affiliations: ¹Department of Genome Modifications and Carcinogenesis, Infection and Cancer Program, German Cancer Research Center (DKFZ), Heidelberg, Germany; and ²International Agency for Research on Cancer, Lyon, France

Corresponding Author: Michael Pawlita, Department of Genome Modifications and Carcinogenesis, Infection and Cancer Program (F020), Deutsches Krebsforschungszentrum (DKFZ), Im Neuenheimer Feld 242, Heidelberg 69120, Germany. Phone: 49-6221-42-4645; Fax: 49-6221-42-4932; E-mail: m.pawlita@dkfz.de

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Materials and Methods

Study population

The study base consisted of 1,022 sexually active women (median age, 36 years; range, 16–63) from a population-based cross-sectional study (overall response rate, 69%) conducted in Ulaanbaatar, Mongolia (2). The aim of the study was to describe HPV prevalence and type distribution in Mongolia to help inform public health choices for cervical cancer prevention in Central Asia. The study was approved by the ethical review committees of the International Agency for Research on Cancer (IARC) and the Mongolian Health Ministry, and informed consent was obtained from all study participants. Paired serum and DBS samples from the same donors were available for 985 women (96%). For the remaining 37 women, paired analyses were not possible because either a valid serum or a valid DBS sample was missing.

Data and sample collection

A standard questionnaire was administered to all study participants by trained interviewers, including questions on age, marital status, and sexual behaviour.

Blood samples were collected between September and November 2005. Venous blood samples for serologic testing (5 mL) were drawn into vacuum containers without anticoagulant. Seventy microliters of full blood each were immediately applied to 5 spots on DBS filter paper cards (Whatman 903 Protein Saver Blood Collection Cards; Schleicher & Schuell), and cards were dried at room temperature. After clotting of the remaining full blood and centrifugation at 2,000 rpm for 5 minutes, 1 mL serum was transferred into a prelabeled 2 mL vial (Safe-lock; Eppendorf). Serum and DBS samples were stored at room temperature for up to 8 hours and then at -20°C for up to 1 month. All samples were shipped at ambient temperature to the German Cancer Research Center (DKFZ) in Heidelberg, Germany, and stored at -20°C until analysis.

Antigens

Serum and DBS samples from study participants were analyzed for antibodies to (i) the major capsid (L1) proteins of the mucosal high-risk HPV types 16 (HPV-16), 18, 31, 33, 45, 52, and 58 (2, 4); (ii) the JCV major capsid protein VP1 (5, 6), (iii) the *H. pylori* proteins NapA (HP0243, strain 26695) and GroEL (HP0010, strain G27; ref. 7); and (iv) the HCV (strain H77, subtype 1a) Core and NS3 proteins (Dondog et al., manuscript submitted).

DBS processing

We assessed suitability of DBS as source for antibodies first by determining the DBS elution volume corresponding to a 1:100 serum dilution. Antibodies against HPV-16 L1, HPV-18 L1, and JCV VP1 in 32 serum/DBS pairs were used for calibration. The best correlation was found for 4 punches with 2.85 mm diameter (corresponding to 15.4% of the spot's area and applied blood volume, respectively,

i.e., 10.8 μL of full blood) eluted in one well of a 96-well plate (Greiner Bio-One polystyrene flat-bottom microplate) overnight at 4°C on a rocker (10 agitations per minute) in 100 μL PBS (data not shown). Sixteen microliters of this eluate correspond to 1.7 μL of full blood and, assuming a red cell content of 45%, 1 μL of serum.

Multiplex serology

The antibody analysis of serum samples was conducted as previously described in detail for HPV (3). Briefly, recombinant viral and bacterial proteins were bacterially expressed as double fusion proteins with N-terminal glutathione *S*-transferase (GST) and a C-terminal peptide (tag) consisting of the last 11 amino acids of the large T antigen of simian virus 40 (GST-X-tag; ref. 8).

GST-X-tag double fusion proteins from cleared bacterial lysates were affinity-purified *in situ* through binding to glutathione casein-coated fluorescence-labeled polystyrene beads. Each fusion protein was bound to a spectrally distinct bead set (SeroMAP Microspheres; Luminex Corp.). Afterward, fusion protein-loaded bead sets were mixed.

Sera were preincubated at 1:50 dilution in serum preincubation buffer consisting of PBS, containing 1 mg/mL casein, 2 mg/mL lysate from bacteria expressing GST-tag without intervening viral or bacterial protein to block antibodies directed against residual bacterial proteins and the GST and tag fusion domains, 0.5% polyvinyl alcohol, 0.8% polyvinyl pyrrolidone, and 2.5% Superchemiblock (Millipore) to suppress unspecific binding of antibodies to the beads themselves (9). Fifty microliters of serum dilutions were incubated with the same volume of mixed bead sets, resulting in a final serum dilution of 1:100.

Sixteen microliters of DBS eluates were mixed with 80 μL DBS preincubation buffer which was identical to the serum preincubation buffer but contained increased concentrations of the relevant background reducing components (i.e., 2.4 mg/mL GST-tag lysate and 3% Superchemiblock) to account for the changed ratio of sample and buffer volume.

Bound antibodies were detected with biotinylated goat-anti-human IgG (H + L) secondary antibody and streptavidin-R-phycoerythrin. A Luminex 100 analyzer (Luminex Corp.) was used to identify the internal color of the individual beads and to quantify their reporter fluorescence [expressed as median fluorescence intensity (MFI) of at least 100 beads per set per serum].

Assay design and data processing

To optimize comparability, serum and DBS eluates of each person were analyzed in parallel on the same 96-well plate and the complete set of samples was tested within 3 consecutive assay days using one batch of antigen-loaded beads. Every day, binding of GST-X-tag double fusion proteins to glutathione casein-coated beads was quantified by monoclonal anti-tag antibody (8). The median interday coefficient of variation (CV) for the anti-tag MFI values for the various antigens was

5.1% (range, 0.1%–19.6%), indicating stable antigen binding to the beads throughout the 3 assay days.

Autofluorescence of each bead set and background reactions resulting from binding of secondary reagents to the antigen-loaded beads were determined in one well per plate without human serum (bead background). The sample background was determined from the reaction of the serum or DBS eluate with beads loaded with GST-tag. Bead background and sample background were subtracted from the raw MFI values obtained with the specific fusion proteins to obtain the antigen-specific reactivity of the sample (net MFI).

Statistical analysis

Serum MFI values for all antigens were categorized as antibody positive or negative by applying previously defined antigen-specific cutoff values (refs. 4, 6, 7; Dondog et al., manuscript submitted). To extrapolate this definition to DBS, cutoff values were calculated to give the same seroprevalence for the DBS as present in the corresponding sera. The cutoff values for sera and DBS are indicated in Table 1.

All statistical analyses were conducted with SAS Version 9.1.3. Correlation of continuous serum and DBS data was assessed by Pearson (R^2) correlation coefficients. For dichotomized data, overall agreement (sum of concordantly positive and concordantly negative samples divided by the total number of samples) and kappa values with

their corresponding 95% confidence intervals (CI) were computed to estimate concordance of serum and DBS results. Categorization of sociodemographic characteristics, computation of OR and their corresponding 95% CI, and tests for linear trends were conducted as described previously (2). All tests were two-sided, and P values below 0.05 were considered statistically significant.

Results

Antigens were classified into 2 groups on the basis of their antibodies' maximum MFI reactivities. The HPV L1 proteins yielded maximum antibody signals below 10,000 MFI (hereafter referred to as "low-titer" antibodies), whereas the *H. pylori*, HCV, and JCV antigens gave maximum antibody signals of up to 30,000 MFI (hereafter referred to as "high-titer" antibodies). The MFI values obtained for 985 DBS/serum pairs for all antigens are illustrated in Fig. 1.

The relationship between the 2 serologic markers followed a slightly J-curved pattern, most evident for the high-titer antibodies (Fig. 1). Overall, the correlation coefficients (R^2) between DBS and serum MFI were high, ranging from 0.72 for HPV-58 L1 to 0.90 for HCV NS3. However, correlation coefficients were better for the high-titer antibodies (median, 0.88; range, 0.80–0.90) than for the low-titer antibodies (median, 0.79; range, 0.72–0.85; Fig. 1). For most antigens, DBS showed

Table 1. Cutoffs and DBS/serum concordance

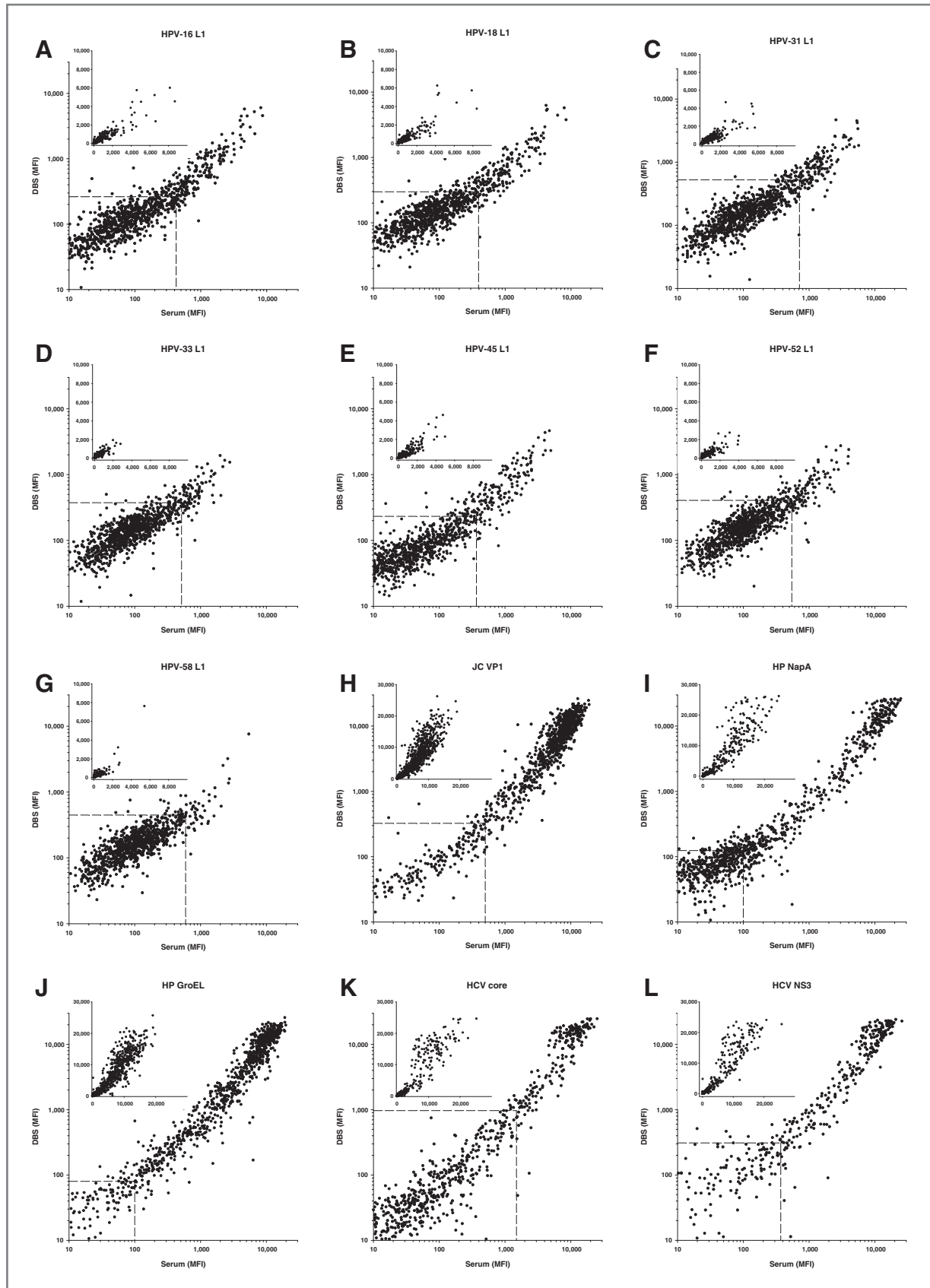
Antigen	Cutoffs (MFI)		Prevalence ^b (%)	Agreement ^c (%)	Kappa (95% CI)
	Sera ^a	DBS			
HPV					
HPV16 L1	422	263	22.4	91.9	0.77 (0.72–0.81)
HPV18 L1	394	298	19.3	93.9	0.80 (0.76–0.85)
HPV31 L1	712	523	13.1	95.1	0.79 (0.73–0.84)
HPV33 L1	515	374	9.2	95.1	0.71 (0.63–0.79)
HPV45 L1	368	232	16.9	94.5	0.80 (0.75–0.85)
HPV52 L1	547	406	10.7	93.9	0.68 (0.60–0.76)
HPV58 L1	592	448	4.8	95.9	0.55 (0.43–0.68)
PyV					
JCV VP1	500	324	78.4	98.0	0.94 (0.91–0.97)
<i>H. pylori</i>					
NapA	100	124	45.5	89.2	0.78 (0.74–0.82)
GroEL	100	80	86.0	96.6	0.86 (0.81–0.91)
HCV					
Core	1,492	967	19.4	98.0	0.94 (0.91–0.96)
NS3	371	310	24.9	96.1	0.90 (0.86–0.93)

Abbreviation: PyV, polyomavirus.

^aSerum cutoff values were predefined in Clifford and colleagues (ref. 4; HPV), Antonsson and colleagues (ref. 6; JCV), Michel and colleagues (ref. 9; *H. pylori*), and Dondog et al., manuscript submitted (HCV).

^bCutoff values for DBS were chosen such that prevalences in serum and DBS were identical.

^c(Concordantly positives + concordantly negatives)/total study population.



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Table 2. Association of sociodemographic characteristics with antibodies to any of the 7 high-risk HPV types as detected by serum and DBS

	Total	Serum		DBS	
		Pos (%)	OR (95% CI)	Pos (%)	OR (95% CI)
Age, y					
<25	204	66 (32.4)	1	71 (34.8)	1
25–34	250	79 (31.6)	0.97 (0.65–1.44)	85 (34.0)	0.97 (0.65–1.42)
35–44	252	96 (38.1)	1.29 (0.87–1.90)	95 (37.7)	1.13 (0.77–1.67)
45–54	212	100 (47.2)	1.87 (1.25–2.78)	91 (42.9)	1.41 (0.95–2.09)
55+	54	26 (48.2)	1.84 (1.06–3.57)	27 (50.0)	1.87 (1.02–3.44)
P_{trend}			0.0001		0.010
Marital status					
Married	691	269 (38.9)	1	262 (37.9)	1
Single	212	62 (29.3)	0.73 (0.48–1.13)	68 (32.1)	0.79 (0.52–1.21)
Widowed	35	19 (54.3)	1.60 (0.80–3.19)	20 (57.1)	2.01 (1.00–4.04)
Separated/divorced	32	17 (53.1)	1.78 (0.87–3.66)	19 (59.4)	2.46 (1.19–5.08)
Lifetime number of sexual partners					
0–1	418	148 (35.4)	1	146 (34.9)	1
2	241	89 (36.9)	1.14 (0.81–1.59)	88 (36.5)	1.12 (0.80–1.56)
3	141	56 (39.7)	1.26 (0.85–1.88)	57 (40.4)	1.31 (0.88–1.94)
4	73	33 (45.2)	1.58 (0.95–2.63)	35 (47.9)	1.80 (1.08–2.98)
5+	81	35 (43.2)	1.45 (0.89–2.37)	37 (45.7)	1.60 (0.98–2.60)
P_{trend}			0.034		0.007

increased minimum reactivities between 30 and 80 MFI. For the low-titer antibodies, MFI in DBS were lower than in serum (median slope derived from linear regression, 0.54; range, 0.50–0.80), whereas for high-titer antibodies, direct comparability of serum and DBS MFI was better (median slope, 1.14; range, 1.09–1.21).

After extrapolation of the predefined serum cutoff values to the DBS, seroprevalences for all antigens in serum and DBS were equal by definition. Numbers of discordant reactions for each antigen were hence symmetrical (e.g., for HPV 16 L1, there were 725 samples concordantly negative, 180 samples concordantly positive, and 40 samples each DBS positive/serum negative and DBS negative/serum positive). Data on serum and DBS seropositivity cutoff values and concordance for all 12 antigens are presented in Table 1.

H. pylori NapA was the only antigen which required a slightly higher cutoff in DBS (124 MFI) than in serum (100 MFI) to give an equal seroprevalence (Table 1). For all other antigens, cutoff points for DBS were lower than for serum (median DBS cutoff 73% of the corresponding serum cutoff), with little variation (range, 62%–84%).

The correlation of the dichotomized DBS and serum data yielded very good agreement (median, 95.1%; range, 89.2%–98.0%) and median kappa values for the low- and high-titer antibodies of 0.77 and 0.90 (range, 0.55–0.80 and 0.78–0.94), respectively, indicating very strong correlations beyond chance. The MFI reactivities of the discordant samples were mostly close to the cutoff (median MFI compared with cutoff, 124%; range, 108%–159%).

The epidemiologic association of risk factors for seropositivity to any of the 7 high-risk HPV types is shown in Table 2 for both serum and DBS. Numbers of positively tested samples were similar for both antibody detection methods. For both serum and DBS, statistically significant associations with seroprevalence were observed for increasing age, increasing number of lifetime sexual partners and by marital status. These associations were equally strong for DBS as for serum. Similar observations were made for increasing age as main risk factor for HCV seropositivity in Mongolian women (Dondog et al., manuscript submitted; data not shown).

Figure 1. Scatter plots of antigen-specific DBS MFI versus serum MFI (985 pairs). Plots for all antigens representing low (A to G) and high (H to L) titer are shown: A, HPV-16 L1 (R^2 , 0.85; slope, 0.52); B, HPV-18 L1 (R^2 , 0.80; slope, 0.54); C, HPV-31 L1 (R^2 , 0.79; slope, 0.59); D, HPV-33 L1 (R^2 , 0.74; slope, 0.53); E, HPV-45 L1 (R^2 , 0.84; slope, 0.54); F, HPV-52 L1 (R^2 , 0.74; slope, 0.50); G, HPV-58 L1 (R^2 , 0.72; slope, 0.80); H, JCV VP1 (R^2 , 0.80; slope, 1.14); I, *H. pylori* (HP) NapA (R^2 , 0.88; slope, 1.09); J, HP GroEL (R^2 , 0.86; slope, 1.12); K, HCV core (R^2 , 0.89; slope, 1.17); L, HCV NS3 (R^2 , 0.90; slope, 1.21). Dashed lines indicate cutoff values as provided in Table 1. Insets in the top left corner of each plot show the same data on linear axes. Please note the maximum values for the insets' axes (10,000 MFI for plots A to G; 30,000 MFI for plots H to L).

Discussion

We directly compared antibody reactivities in 985 pairs of DBS on filter paper cards and serum samples derived from intravenous blood to explore the feasibility of DBS-based seroepidemiologic studies. The main strengths of the present study are the large number of almost 1,000 sample pairs tested in parallel, the use of state-of-the-art technique for detection of multiple antibodies, and the integration of 12 different antigens from 4 groups of infectious agents representing both low- and high-titer antibody responses: HPV, *H. pylori*, HCV, and JCV.

The infectious agents involved in this study are known to cause different diseases. The mucosal high-risk HPV types 16, 18, 31, 33, 45, 52, and 58 are causally associated with several malignancies, particularly cervical cancer (10). *H. pylori* infection is related to severe gastrointestinal diseases including gastric cancer (11), and chronic HCV infection may lead to hepatocellular carcinoma and non-Hodgkin lymphoma (10). Finally, JCV can cause progressive multifocal leukoencephalopathy in immunosuppressed patients (12). Despite this heterogeneity, antibody detection is valuable for each of these infectious agents, to assess their prevalence and to discover yet unknown disease associations in seroepidemiologic studies (13). Our multiplex serology method was first developed for HPV serology (3) and the versatile bacterial protein expression system has facilitated the method's extension to antigens from other infectious agents. Multiplex serology-based polyomavirus (5, 6) and *H. pylori* serology (7) has also been reported. Furthermore, the application of multiplex serology to both HIV (Kranz et al., manuscript submitted) and HCV (Dondog et al., manuscript submitted), including validation against gold-standard serologic tests, will soon be available in the published literature.

The seroprevalences of *H. pylori* (14) and JCV (5, 6) are quite high in adults of most populations, whereas antibodies to mucosal HPV (15) and HCV (16) are less abundant and variable by population. Mongolia is a high-risk country for infections with both mucosal HPV (2) and HCV (17) which makes our study population especially well suited for this comparison of serologic methods. We included 2 exemplary *H. pylori* antigens with high (GroEL) and low (NapA) seroprevalence; these do, however, not allow assessing overall *H. pylori* seroprevalence. Our data on the overall prevalence of *H. pylori* in serum samples [81% as determined by *H. pylori* multiplex serology using all 15 antigens (ref. 7; unpublished observation) and JCV (78%, this report)] in Mongolian adults are in concordance with published data (18, 19).

DBS have been mainly used for viral or bacterial infections with high antibody titers such as the HIV, hepatitis B, and Measles viruses, or the bacterium *Mycobacterium leprae* (reviewed in ref. 1). HCV is the only of the 4 infectious agents analyzed here for which DBS have been used before (e.g., ref. 20), but to our knowledge, there is no published DBS data available for *H. pylori*, JCV, or HPV. We considered the application of DBS for HPV serology as

the main challenge, as because of the absence of systemic infection, natural HPV antibody titers are low. However, HPV titers after vaccination are much higher than after natural infections (21), facilitating the use of DBS in HPV vaccination studies.

Our results indicate that overall quantitative DBS and serum results are well comparable. However, we observed deviation from the ideal (linear) DBS/serum correlation. For very low antibody reactivities (~100 net MFI, i.e., after background subtraction), DBS yield higher baseline reactions, whereas for intermediate reactivities (~1,000 MFI), DBS generate lower MFI than serum. This drives the overall correlation of the low-titer antigens representing mostly weak and intermediate antibody reactivities to slopes below 1, that is, overall lower DBS than serum values. Strong antibody reactivities (~10,000 MFI) however yield similar MFI values in serum and DBS, resulting in a curved correlation as seen for the high-titer antigens. At present, we can only speculate about the causes underlying these phenomena. Baseline elevation in DBS could result from highly abundant, eluted full blood components not present in serum or plasma (e.g., hemoglobin) which mediate unspecific immunoglobulin binding to the viral or bacterial proteins used as antigens. The curved correlation could result from small concentrations of inhibitors or competitors competing with antibody binding to the antigens. With higher titers of antigen-specific antibodies in the DBS eluates, both phenomena are increasingly overcome.

In the presence of paired serum and DBS samples, we were able to extrapolate predefined serum cutoff values to the DBS data, by choosing DBS cutoff values that resulted in identical seroprevalence estimates, a key outcome in epidemiologic studies. This method resulted, by definition, in symmetric discordance and was more robust against individual outliers than regression-based methods (data not shown). After application of the extrapolated cutoff values, kappa values for DBS/serum concordance were high for the antigens with high-titer antibodies (with a median of 0.90), thus approaching values that are found for reproducibility of multiplex serology (typically ≥ 0.95). Overall concordance was still good for the HPV antigens, with the exception of HPV-58 with a kappa value of 0.55. However, kappa statistics are strongly influenced by prevalence, and HPV-58 was the type with the lowest seroprevalence (<5%). For all antigens, antibody reactivities of the discordant samples were mostly close to the respective cutoff value. We therefore believe that discordance was largely a stochastic event. Indeed, epidemiologic associations of established risk factors for HPV (2) and HCV (Dondog et al., manuscript submitted) seropositivity in Mongolia were shown to be equally strong for DBS as for serum.

The availability of paired data from both methods is an exception. Purely DBS-based studies will rely on simultaneous collection of samples from appropriate control groups to define internally valid cutoff values (e.g., children and/or virgins when studying sexually

transmitted diseases). Alternatively, in the absence of such controls, our data also suggest how existing cutoff values established in serum may be extrapolated to DBS. For 11 of 12 antigens, we found the DBS cutoff values to be approximately 73% of the corresponding serum cutoff values, within a relatively narrow range of $\pm 11\%$, probably owing to random variation. A limitation of the DBS method is that standardized reference sera cannot be used.

DBS facilitate field work in epidemiologic studies especially in low resource countries because blood does not need to undergo centrifugation and the DBS cards can be stored and shipped at ambient temperature. Blood sampling procedures may also be facilitated, as sufficient amounts of blood for DBS preparation from adults and even children may be obtained from the finger tip, the earlobe, or the heel as done routinely with diabetes patients and newborns, thereby strongly reducing costs of seroepidemiologic studies. Of note, however, we used blood drops from intravenous puncture to prepare DBS, which facilitates high quality and reproducibility of DBS.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

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