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Comparison of available methods to elute serum from dried blood spot samples for measles serology☆

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Abstract

Six existing protocols for the extraction of serum from blood spots dried onto filter paper were compared. Assessment criteria included: detection of measles IgM and IgG by the Dade Behring Enzygnost® immunoassays, volumes of recovered eluates, reproducibility, processing time and throughput, difficulty of protocol, equipment required, safety and estimated costs. Detection of measles IgM in eluates obtained by four of these protocols was as in serum, and significant differences were only observed in eluates from the two remaining protocols ($p < 0.05$). Significant differences were found between extraction protocols regarding measles-specific IgG detection when an IgG indeterminate DBS was analyzed ($p < 0.05$), but not when an IgG positive and negative DBS were studied. Sufficient eluate volumes were recovered for testing in the IgM Behring assay following all protocols but two. Sufficient eluate was recovered for testing in the IgG Behring assay following all six protocols. While all protocols were relatively easy to perform, only two protocols required less than 2 h for completion. In general, compared protocols performed well on the extraction of antibodies from DBS for serology with differences being observed with eluate volume recovery, turn around time, required equipment and cost. An easy-to-implement protocol is proposed for the rapid extraction of serum for measles/rubella serology in outbreak situations for use in the World Health Organization Global Measles and Rubella Laboratory Network.

Keywords: Filter paper blood spot; Enzyme immunoassay; IgM detection; IgG detection; Protocol; Elution

1. Introduction

Measles remains a serious vaccine-preventable disease, and is estimated to be responsible for more than half a million childhood deaths worldwide (World Health Organization, 2005). Laboratory surveillance coupled with epidemiological surveillance is essential to guide measles control efforts in the quest for reducing global measles mortality and achieving regional measles elimination goals. Three key laboratory activities are (1) confirmation of measles suspected cases, (2) description of population seroprevalence profiles through serology, and (3) verification of measles virus circulation through genetic characterization (Featherstone et al., 2003). Serum is the specimen most frequently used for measles serology, while throat swabs, urine and/or whole blood are collected for genetic characterization. Thus, proper suspect case investigation requires that several specimens be obtained. However, these specimens can pose problems in: (1) their collection, i.e., the invasive nature of venipuncture and its poor acceptance by the patient and parents, and (2) their storage and transportation, i.e., maintenance of the specimens at the site of collection and reverse cold chain from hard-to-reach areas to testing locations (Bellini and Helfand, 2003).

The World Health Organization (WHO) supports the use of oral fluid and filter paper dried whole blood spots (DBS) as specimen collection alternatives to overcome the abovementioned limitations. Inclusion of oral fluid and DBS in the list of specimens collected for measles will facilitate collection and transport of specimens, as well as offer the potential for performing serology and genetic characterization using a single sample (Helfand

☆ The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the U.S. Department of Health and Human Services. Use of trade names and commercial sources is for identification only and does not imply endorsement by the U.S. Department of Health and Human Services.

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et al., 2001; Nigatu et al., 2001; Nokes et al., 2001). The advantages of collecting DBS have been well-documented elsewhere (Mei et al., 2001; Parker and Cubitt, 1999). Recent performance evaluation studies using commercially available and in-house enzyme immunoassays, demonstrated that collection of DBS is a feasible and reproducible alternative to phlebotomy for measles diagnostics (Chakravarti et al., 2003; De Swart et al., 2001; el Mubarak et al., 2004; Helfand et al., 2001; Riddell et al., 2003, 2002). Similar studies have been performed and are currently underway for rubella serology (Condorelli et al., 1994; Helfand et al., 2001; Karapanagiotidis et al., 2005). Amplification of measles virus RNA from DBS by reverse transcriptase PCR and subsequent sequence analysis has also shown promising results (De Swart et al., 2001; el Mubarak et al., 2004; Katz et al., 2002; Mosquera et al., 2004). This paper is a collaborative pursuit with the World Health Organization to establish a standardized method to recover serum from DBS for measles serology.

Several methods have been used to extract blood products including serum from DBS for serology of infectious diseases, among them measles and rubella. Some of the protocols are ideal for field situations, while others require well-equipped laboratories. In all protocols, discs are excised from DBS and soaked in elution buffer, which helps to diffuse serum components from the rehydrated filter paper matrix (Chanbancherd et al., 1999; Condorelli et al., 1994; el Mubarak et al., 2004; Farzadegan et al., 1978; Fortes et al., 1989; Hanna et al., 1989; Helfand et al., 2001; Hogrefe et al., 2002; Lindhardt et al., 1987; Pannuti et al., 1991; Parker et al., 1995; Steger et al., 1990; Tappin et al., 1998; Varnier et al., 1988; Vejtorp and Leerhoy, 1981). In some instances, serum diffusion is integrated in the immunoassay procedure (Condorelli and Ziegler, 1993; Eaton et al., 1996; Kalter et al., 1992; Neto et al., 2004). In other protocols, DBS serum elution is taken one step further by manually extruding or by centrifuging the soaked discs to actively recover DBS components (Brody et al., 1964; Chishty, 1971; De Swart et al., 2001; Khare et al., 1985; Monto et al., 1969; Nakano et al., 1983; Riddell et al., 2002; Saha and Saxena, 1983). Additionally, protocols may differ in the number and diameter of discs for elution, depending on the subsequent serological immunoassay (Chanbancherd et al., 1999; Chishty, 1971; Condorelli et al., 1994; De Swart et al., 2001; Draper and Kelly, 1969; el Mubarak et al., 2004; Fortes et al., 1989; Hanna et al., 1989; Helfand et al., 2001; Hogrefe et al., 2002; Khare et al., 1985; Mosquera et al., 2004; Pannuti et al., 1991; Parker et al., 1995; Riddell et al., 2003; Saha and Saxena, 1983; Tappin et al., 1998).

The goal of this paper is to compare available methods for DBS serum extraction for measles serodiagnosis and propose one that will permit the recovery of the maximum volume of eluted sample in the minimum time, effort and cost. We compared six protocols using the measles Dade Behring Enzygnost® immunoassays which are widely used in the WHO Global Measles and Rubella Laboratory Network. The paper attempts to provide a general overview of available methods to elute serum from blood samples dried on filter paper and proposes an extraction method suitable for general use in the Global Measles and Rubella Laboratory Network.

2. Materials and methods

2.1. Literature review

PubMed from the United States National Center for Biotechnology Information was utilized to identify publications which described methods to elute DBS for serodiagnosis.

2.2. Samples

A panel of five simulated DBS samples was prepared and consisted of an IgM positive, an IgM negative, an IgG positive, an IgG equivocal and an IgG negative DBS. These DBS samples were prepared at the Centers for Disease Control and Prevention (CDC, Atlanta, USA) using serum of known IgM and IgG antibody optical density values (as tested by the measles Dade Behring Enzygnost® immunoassays) and washed red blood cells as described elsewhere (Merideth and Hannon, 1993). The sample hematocrit was adjusted to 50%. Negative controls consisted of (1) red blood cells and saline instead of serum, (2) red blood cells and antibody negative serum and (3) a control made of plain filter paper. Before testing, the quality of the DBS was determined by visual inspection (NSPWC, 2003).

2.3. Elution of serum from DBS

A step-by-step description of each of the six compared protocols is provided in Table 1. Protocols 1, 2, and 5 were adapted to the Dade Behring Enzygnost® Anti-Measles Virus IgM and IgG enzyme immunoassays (Marburg, Germany) as follows. First, the required number of dried blood filter paper discs to satisfy the manufacturer's specifications regarding serum dilution (1:21) was calculated by making the assumption that a 6.35 mm (1/4 in.) filter paper disc contained about 6 μL of dried serum (Mei et al., 2001). Second, the volumes of elution buffer used per extraction were 250 and 125 μL per sample for IgM and IgG antibody detection, respectively. Hence, two 6.35 mm discs were removed from each DBS sample using a hole perforator to prepare the extracts for IgM antibody detection, while a single disc of the same size was sufficient for IgG antibody detection. Third, the DBS elution buffer composition was phosphate buffered saline containing Tween 20 and 5% non-fat dry milk (Condorelli et al., 1994; Helfand et al., 2001; Riddell et al., 2003, 2002). This elution buffer has been reported to reduce background interference in the detection of measles antibodies in DBS sample eluates by enzyme linked immunosorbent assay (ELISA) (Condorelli et al., 1994). Protocol 4 is a modification of protocol 3, where discs were soaked in elution buffer in microcentrifuge tubes instead of 96-welled plates (Table 1) (Akoua-Koffi, 2004; Korukluoglu, 2004; Riddell et al., 2002, 2003). In protocols 3 and 4, elution buffer volumes of 220 and 250 μL per sample were used for IgM and IgG antibody detection as described elsewhere (Riddell et al., 2002, 2003). Finally, protocol 6 was developed at CDC and was performed as follows. Two 6.35 mm diameter discs were excised from DBS, and were placed in labeled 24-welled plates. Discs were soaked in 250 μL of elution buffer, covered with a lid and incubated for 30–60 min

Table 1
Step-by-step protocol description

	Protocols					
	1	2	3	4	5	6
Reference	Helfand et al. (2001), Fortes et al. (1989), Hogrefe et al. (2002)	Condorelli et al. (1994), Hanna et al. (1989), Tappin et al. (1998)	Riddell et al. (2002), Riddell et al. (2003)	Korukluoglu (2004), Akoua-Koffi (2004)	Draper and Kelly (1969), Chishty (1971), Monto et al. (1969)	
Elution container	96-Well plate	96-Well plate	96-Well plate	Microcentrifuge tube	Syringe barrel	48-well plate
1. Punch DBS[a] disc 2. Add elution buffer	R[b,c]	R[c]	R[d]	R[d]	R[c]	R[c]
3. Agitate on an automatic shaker	–[e]	30 min	15 min	15 min	–	–
4. Incubate	o/n[f] 4 °C	o/n 4 °C	o/n 4 °C	o/n 4 °C	1 h RT[g]	1 h RT
5. Agitate	–	–	15 min	15 min	–	–
6. Transfer disc/buffer into column	–	–	–	–	–	R
7. Apply force	–	–	10 min 2200 × *g*[h]	10 min 2200 × *g*	Manual	30 min 1800 × *g*
8. Remove eluate from container with a pipette	R	R	R	R	–	–
9. Test/store sample	R	R	R	R	R	R

[a] Dried blood spot.
[b] R: step required in protocol.
[c] Two discs and 250 μL/sample of elution buffer for Dade Behring Enzygnost® anti-measles IgM testing; 1 disc and 125 μL/sample of elution buffer for Dade Behring Enzygnost® anti-measles IgG testing.
[d] Two discs and 220 μL/sample of elution buffer for Dade Behring Enzygnost® anti-measles IgM testing; 1 disc and 250 μL/sample of elution buffer for Dade Behring Enzygnost® anti-measles IgG testing testing.
[e] Step not required in protocol.
[f] Overnight as 16 h.
[g] Room temperature.
[h] Relative centrifugal force.

at room temperature. Following incubation, a pipette tip was used per sample to transfer soaked discs and elution buffer into the corresponding centrifuging system. The centrifuging system for simultaneous elution for IgM and IgG testing consisted of a labeled 10 mL centrifuge tube that held a microtube (Bio-Rad Titertube®), and supported an uncapped 3 mL syringe barrel at the open end (Fig. 1). Samples were centrifuged at room temperature for 30 min at 1800 × *g* relative centrifugal force in a Thermo IEC Centra CL3R centrifuge. Eluate-containing microtubes were removed from inside the centrifuge tube using a pre-cut 1250 μL pipette tip that fit tightly inside the microtube. Microtubes were then placed in testing racks, and were stored at −20 °C until used. The microtube was used in the simultaneous elution for IgM and IgG testing to minimize loss of eluate volume through pipetting. However, the microtube was not strictly required for elution for IgM testing alone, since ample eluate volume was recovered. When the microtube was not required, the eluate was collected in the labeled 10 mL centrifuge tube.

2.4. Commercial IgM indirect enzyme immunoassay

DBS eluted by all six protocols were tested on the Dade Behring Enzygnost® Anti-Measles Virus IgM enzyme immunoassay (Marburg, Germany) per the manufacturer's instructions, with the following modifications indicated per protocol. The reactivity of IgG and rheumatoid factor (RF) was blocked by the addition of RF absorbent as per manufacturer's instructions. In protocols 1, 2, 5, and 6, 160 μL of the absorbent was added to each eluted sample, whereas in protocols 3 and 4, 170 μL of the absorbent was added to each eluted sample, as described elsewhere (Riddell et al., 2002). When less than 170 μL of sample was recovered, i.e., following protocols 3 and 4 (see Table 2 for mean recovered volume ± standard deviation and median), sufficient RF absorbent volume was added to bring the final volume to 340 μL. As per kit instructions, a volume of 150 μL of this mixture was loaded onto the antigen-coated plate. The final sample dilution in each well was approximately 1:44. For sample eluates of all six protocols, the rest of the testing procedure was as described in the insert supplied with each assay kit. Protocols 3 and 4 were also assayed by the modified conditions described elsewhere, with extended incubation times (1.5 h instead of 1 h) and number of washes (5 instead of 4) (Riddell et al., 2002). Serum samples that were used to prepare the mock DBS samples were tested in parallel with the DBS following the kit insert instructions. Assay validation, calculation of final optical density values and result classification were performed as described in the insert. No measurement correction was performed on delta values from DBS eluates and sera, since it remains unknown how the serum-based EIA values used

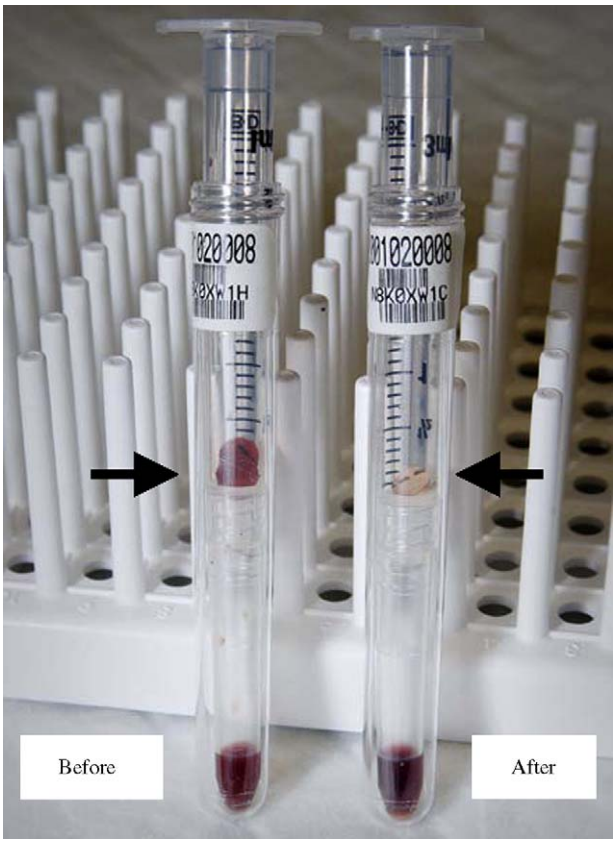

Fig. 1. Column centrifugation systems before and after centrifugation of dried blood spot sample. Arrows point at filter paper discs, showing appearance before and after centrifugation.

for the calculation of the correction factor relate to DBS eluate samples.

2.5. Commercial IgG indirect enzyme immunoassay

DBS eluates obtained by protocols 1 through 6 were tested using the Dade Behring Enzygnost® Anti-Measles Virus IgG enzyme immunoassay (Marburg, Germany) per the manufacturer's instructions, including assay validation, calculation of final optical density values and result classification. The only modification was that 50 μL of eluates from protocols 3 and 4 were added to wells containing 170 μL of sample buffer (Riddell et al., 2003). Serum samples that were used to prepare the mock DBS samples were also assayed per Dade Behring's instructions, but not in parallel with the DBS. No measurement correction was performed on delta values from DBS eluates and sera.

2.6. Data analysis

Protocols 1 through 6 were compared using criteria that included antibody detection, eluted DBS sample recovery, protocol difficulty, processing capabilities, required time and equipment, safety, and cost. Each protocol was repeated up to six times and eluates from all protocols were assayed in parallel by the measles IgM and IgG Dade Behring assays for up to five different runs. For IgM detection, cognate serum specimens were run each time in parallel with the eluates. Nonparametric statistics were used for analysis, because the data may not be normally distributed (Lehmann, 1975). Elution protocols for IgM detection on positive and negative DBS eluates were compared to the cognate serum by using the Wilcoxon rank-sum test. Elution protocols for IgG detection on positive, intermediate, and negative DBS eluates were each compared by using the Kruskal–Wallis test. The significance level was <0.05. Statistical analysis was performed using SAS 9.1.

3. Results

3.1. Selection and comparison of DBS elution protocols

A PubMed literature search led to the identification of 74 articles where DBS were collected for serodiagnosis of infectious diseases. Although the methods of DBS serum extraction varied from paper to paper, four core protocols were recognized. For reference purposes, these protocols were labeled as protocol 1, 2, 3, and 5 (Table 1). For laboratories without an ELISA plate centrifuge, protocol 3 was modified and is referred to as protocol 4 (Table 1). Protocol 6 was developed at CDC (Table 1). In protocols 1 and 2, blood components were passively eluted from DBS samples without any applied force, while in protocols 3 through 6 a force (centrifugal or manual) was applied to actively elute the samples.

Protocols 1 through 6 were compared to provide an overview of available methods by both passive (protocols 1 and 2) and active (protocols 3 through 6) extraction. Protocols 1–4 were similar in that 96-welled plates or microcentrifuge tubes were used to soak the DBS discs and the eluted sample was removed from the vessel with a pipette. The difference among these four protocols was the use of instruments to facilitate specimen transfer. In contrast, protocols 5 and 6 differed in that both used a syringe barrel as a means of sample recovery. In protocol 5, following incubation with elution buffer, the syringe plunger was pressed down the column and the resultant pressure expressed the hydrated specimen from the filter paper and into a labeled collecting tube. In protocol 6, DBS discs were soaked in a 24- or 48-welled plate, syringe columns containing soaked discs and partially eluted samples were centrifuged and the generated centrifugal force efficiently removed the hydrated specimens from the discs into the labeled tubes (Fig. 1). The six protocols were compared using the criteria presented in Tables 2–7. Tables 2–4, 6 and 7 summarize the findings related to protocol performance (antibody detection and final eluted sample volume), as well as time needed to perform the protocol and cost. Table 5 identifies within each protocol different practical features and presents required equipment. The studied protocols were modified so that the recovered samples from DBS could subsequently be tested in the aforementioned commercial immunoassays.

3.2. Antibody detection in DBS eluates

The ability to detect measles IgM antibodies in samples eluted from DBS under the six protocols was determined using DBS

Table 2
Volume of recovered dried blood spot eluate

Protocol	Elution for IgM detection[a]		Elution for IgG detection[a]	
	Mean volume (S.D.)[b]	Median	Mean volume (S.D.)	Median
1	200 (5.50)[c,d]	200.0	98 (7.12)[c,e]	100.0
2	195 (9.43)[c,d]	192.5	93 (6.61)[c,e]	95.0
3	158 (8.90)[f,g]	162.5	206 (7.41)[c,h]	210.0
4	168 (5.87)[f,g]	167.5	215 (6.55)[c,h]	215.0
5	205 (13.12)[c,d]	210.0	67 (10.75)[c,e]	60.0
6	215 (7.82)[c,d]	215.0	101 (5.27)[c,e]	100.0

[a] Number of samples measured per protocol is 10.
[b] Optical density expressed as mean optical density values with standard deviation in parenthesis.
[c] Enough volume was recovered for testing.
[d] Assay requires 160 μL of eluate sample.
[e] Assay requires 40 μL of eluate sample.
[f] Not enough volume was recovered for testing.
[g] Assay requires 170 μL of eluate sample.
[h] Assay requires 100 μL of eluate sample.

constructed with IgM positive and IgM negative serum specimens. No significant differences were observed when detection of measles virus IgM in DBS eluates extracted by protocols 3 through 6 (active elution methods) was compared to detection in the corresponding serum used to prepare the DBS (Fig. 2 and Table 3). However, IgM antibody detection was found to be significantly different from serum ($P \leq 0.05$) in (a) eluates from protocols 1 and 2 obtained by passive elution, where optical density values were lower than in serum (Fig. 2, panel A) and (b) in eluates from protocols 3 and 4 assayed by the modified version of the Dade Behring enzyme immunoassay (Fig. 2, panel C) (Riddell et al., 2002). The latter resulted in higher optical density values, which were likely due to the extended incubation periods (1.5 h instead of 1 h). Similarly, detection of IgG antibodies was studied using DBS prepared with sera classified as IgG positive, equivocal and negative. No significant differences in IgG detection were found between protocols in the positive and negative DBS samples (Fig. 3 and Table 4). In contrast, significant differences were found in the IgG equivocal DBS sample ($P \leq 0.05$). In the latter, all measurements performed on eluates from protocols 2, 5 and 6 fell within the defined 0.100–0.200 interval for an equivocal sample. However, 1 in 5 EIA measurements on eluates from protocols 3 and 4 where higher than the 0.200 upper cut-off value of the aforementioned interval, while one outlier was detected in eluates from protocol 1 (Fig. 3 and Table 4).

IgM detection reproducibility and detection rates were excellent for all eluate samples recovered using the six protocols. A low standard deviation and 100% concordance was observed when eluted DBS specimens were compared with the cognate serum specimens (Table 3). IgG detection rates on the positive DBS eluted samples were excellent regardless of elution protocol, however equivocal DBS samples eluted with protocols 1, 3 and 4, had detection rates of only 80% (Table 4). All eluated specimens obtained using all protocols yielded an EIA result that was classified as positive, as expected for a DBS prepared with an IgG positive control serum. All specimens obtained from the IgG equivocal DBS were classified as equivocal, except for three that were positive. These three were eluted following protocols 1, 3 and 4. Eluted samples from DBS prepared with an IgG negative serum yielded the expected negative result. In general, IgG detection reproducibility was very good, except for protocol 1, where the coefficient of variation was 35%.

Table 3
Detection of IgM anti-measles virus antibodies in dried blood spot samples and cognate serum by the Dade Behring Enzygnost® assay expressed as optical density values and percentage detection rates

Protocol	IgM detection on IgM positive dried blood spot eluate[a]		
	Optical density (S.D.)[b]	Median	Detection rate (%)[c]
1[d]	0.455 (0.034)[e]	0.467	100 (5/5)
2[d]	0.456 (0.020)[e]	0.453	100 (5/5)
3[d]	0.478 (0.026)[f]	0.477	100 (5/5)
4[d]	0.484 (0.024)[f]	0.484	100 (5/5)
3[g]	0.648 (0.041)[e]	0.654	100 (5/5)
4[g]	0.649 (0.030)[e]	0.666	100 (5/5)
5[d]	0.464 (0.018)[f]	0.454	100 (5/5)
6[d]	0.477 (0.018)[f]	0.479	100 (5/5)
Serum	0.482 (0.008)	0.482	n/a[h]

[a] Number of elution and antibody detection repeats is 5.
[b] Optical density expressed as mean optical density values with standard deviation in parenthesis.
[c] Number of eluate samples with *P* result/ number of repeats.
[d] IgM detection by Dade Behring Enzygnost® assay per manufacturer's instructions.
[e] Optical density significantly different from serum ($p \leq 0.05$).
[f] Optical density not significantly different from serum.
[g] IgM detection by Dade Behring Enzygnost® assay per modified instructions (Riddell et al., 2002).
[h] Not applicable.

Table 4
Detection of IgG anti-measles virus antibodies in dried blood spot samples by the Dade Behring Enzygnost® assay expressed as optical density values and percentage detection rates

Protocol	IgG positive dried blood spot eluate[a]		
	Optical density (S.D.)[b,c]	Median	Detection rate[d], %
1	1.478 (0.137)	1.469	100 (5/5)
2	1.496 (0.148)	1.497	100 (4/4)
3	1.468 (0.169)	1.504	100 (5/5)
4	1.526 (0.154)	1.554	100 (6/6)
5	1.517 (0.010)	1.517	100 (2/2)
6	1.431 (0.161)	1.428	100 (6/6)
Protocol	IgG equivocal dried blood spot eluate[a]		
	Optical density (S.D.)[e]	Median	Detection rate, %
1	0.153 (0.053)	0.137	80 (4/5)
2	0.134 (0.018)	0.126	100 (5/5)
3	0.175 (0.024)	0.174	80 (4/5)
4	0.178 (0.020)	0.167	80 (4/5)
5	0.130 (0.008)	0.130	100 (2/2)
6	0.136 (0.011)	0.138	100 (5/5)

[a] Number of elution and antibody detection repeats is ≤5.
[b] Optical density expressed as mean optical density values with standard deviation in parenthesis.
[c] Optical density values not significantly different between protocols.
[d] Number of eluate samples with *P* (or *E*) result/number of repeats.
[e] Optical density values significantly different between protocols ($p \leq 0.05$).

Table 5
Comparison of practical and logistic aspects of evaluated protocols

	Protocols					
	1	2	3	4	5	6
Easy to perform	x[a]	x	x	x		x
Fast (less than 3 h)					x	x
Minimal material preparation	x	x	x	x		
No electricity required	x				x	
Required equipment/materials easily found in serology labs	x				x	x
Low cost and easy-to-implement in serology laboratories	x				x	x
High sample throughput	x	x	x			
Eluate sample volume is completely extracted from filter paper					x	x
Eluate sample volume is adequate for the Dade Behring IgM assay	x	x			x	x
Eluate sample volume is adequate for the Dade Behring IgG assay	x	x	x	x	x	x
Good reproducibility in eluate volume recovery	x	x	x	x		x
Labeling is easy				x	x	x
Minimal risk of sample misplacement/cross contamination				x	x	x
Minimal risk for sample aerosolization	x	x	x		x	x
Required equipment						
Microplate shaker		x	x			
2 mL tube shaker				x		
Microplate centrifuge			x			
2 mL tube centrifuge				x		
15 mL tube centrifuge						x
Required disposables						
Hole perforator	x	x	x	x	x	x
96-Welled plate	x	x	x			
Microcentrifuge tube				x		
1 mL syringe					x	
24-Welled plate						x
3 mL syringe						x
10 mL centrifuge tube						x
Microtiter tube						x[b]
1 mL pipette tip						x[b]
0.3 mL pipette tips	x	x	x	x	x	x

[a] x: applies to protocol.
[b] Only required to simultaneously elute samples for IgM and IgG antibody detection. Not required to elute sample for IgM antibody detection alone.

3.3. Volume of recovered DBS eluate

Protocol performance based on volume of recovered specimen was found to be highly reproducible in all procedures, except for protocol 5 which, due to handling during manual extraction, had the highest standard deviation in sample recovery (Table 2). For testing on the anti-measles Dade Behring IgM assay, addition of 250 μL of elution buffer per extraction was found to yield ample specimen for testing. Elution by protocols 3 and 4 with 220 μL of elution buffer resulted in suboptimal volumes of recovered specimen. It was difficult to recover the required 170 μL for testing. Therefore, an eluate volume smaller than recommended was used only for the purpose of this comparison. Such modification in the protocols should not be applied for diagnostic purposes. In contrast, specimen recovery using protocol 6 resulted in sufficient sample volume to allow testing for both IgM and IgG Dade Behring assays. For IgG detection, elution of specimens from DBS in either 125 μL (protocols 1, 2, 5 and 6) or 250 μL (protocol 3 and 4) also resulted in adequate volumes of recovered specimen. Of all methods, protocols 5 and 6 were those that resulted in what appeared to be complete removal of blood products from the DBS since the resultant filter paper discs were practically white and dry after the procedure (Fig. 1). In contrast, filter paper discs following protocols 1–4 were seemingly saturated with hydrated blood, indicating that a portion of the eluted sample was trapped in the filter paper fibers, and between the filter paper discs and could not be recovered.

Table 6
Turn around time per protocol

Protocol	Processing time
1	o/n[a] + 45 min
2	o/n + 1.5 h
3	o/n + 2.5 h
4	o/n + 1.5 h
5	1.5 h
6	2 h

[a] Overnight as 16 h.

Table 7
Estimated cost per protocol per DBS[a] and per 96 DBS samples

Protocol	Per DBS[b] ($)	Per 96 DBS[c] ($)
1	1.69	0.05
2	1.69[d]	0.05[d]
3	1.69[e]	0.05[e]
4	0.06[f]	0.06[f]
5	0.14	0.14
6	1.02[g]	0.40[g]

[a] Dried blood spot.
[b] Cost to process 1 DBS sample.
[c] Cost to process 96 DBS samples.
[d] Add cost of an orbital shaker.
[e] Add costs of an orbital shaker and a centrifuge with 96-well plate adapters.
[f] Add costs of an orbital shaker and a centrifuge for microcentriguge tubes.
[g] Add cost of a centrifuge for serology tubes.

3.4. Logistic and practical aspects

In general, all six protocols were found to be relatively easy and safe to perform (Table 5). Protocol 5 was less straightforward due to manual handling during elution. Protocols 4–6 were found to be somewhat more laborious due to material preparation (protocols 5 and 6), and due to handling of microcentrifuge tubes (protocol 4). Protocol 4 was found to be relatively less safe due to the potential of sample aerosolization when opening and closing the microcentrifuge tubes. Protocols that do not require any electric powered equipment are obviously more suitable in field situations, where infrastructure may be limited (protocols 1 and 5). Protocols that do not require overnight DBS elution were found to be suitable in outbreak situations, when rapid processing of small numbers of samples is needed (protocol 5 and 6) (Table 6). Protocols using 96-welled plates and that require overnight incubation were found suitable for large stud-

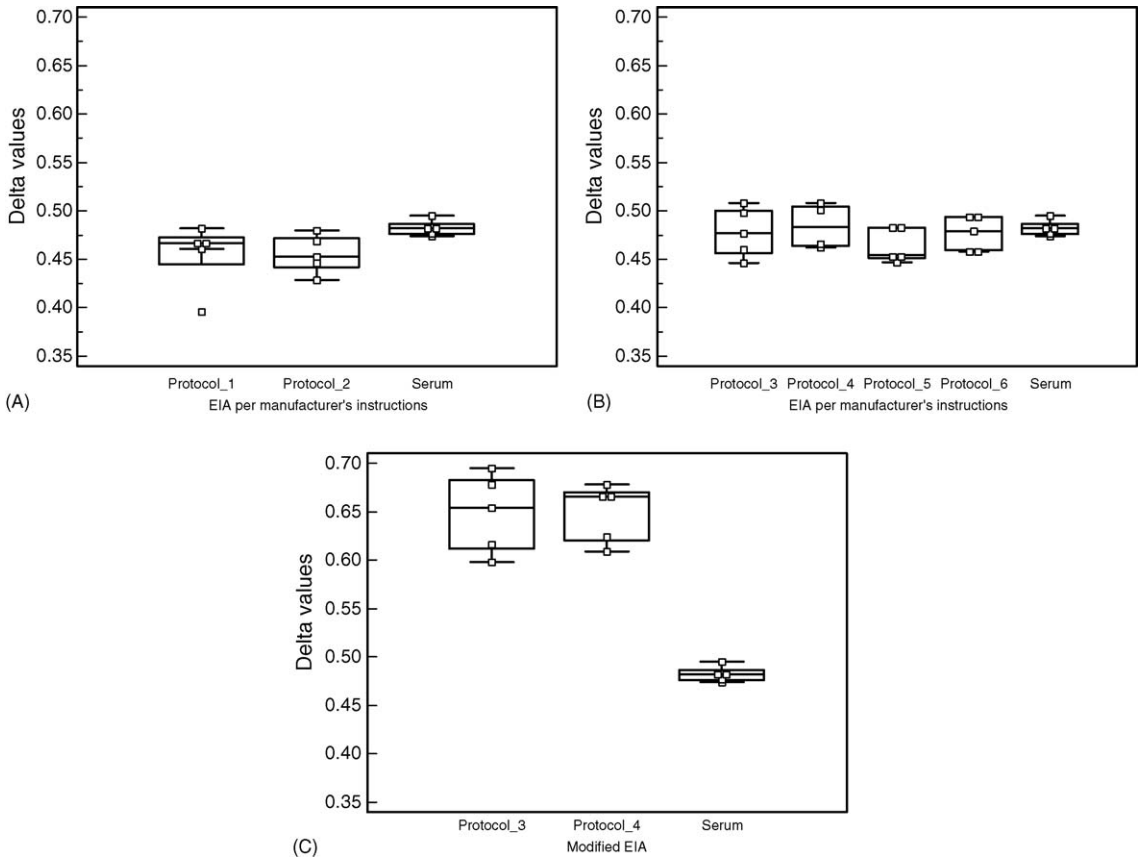

Fig. 2. Detection of antibody by the anti-measles virus IgM Dade Behring EIA after elution of an IgM positive DBS sample by six different extraction protocols and compared to detection in serum. An IgM positive DBS was eluted up to five times per each extraction protocol. Eluates resulting from the six extraction protocols were tested in parallel in five separate runs. Panel A: delta values from eluates from passive elution protocols 1 and 2 tested per manufacturer's instructions were significantly different from serum ($p \leq 0.05$). Panel B: delta values from eluates from active elution protocols 3–6 tested per manufacturer's instructions were not significantly different from serum. Panel C: delta values from eluates from active elution protocols 3 and 4 per modified assay conditions were significantly different from serum ($p \leq 0.05$). Delta value is the optical density value of the positive well minus the optical density value of the negative well. Assay cut-off value for a positive sample is a delta value of 0.200. *Y*-axis minimum value is set at 0.350.

ies when high throughput of samples would be required, but use of 48-welled plates would probably work better for more easy handling of DBS discs, labeling and minimizing sample cross-contamination (protocols 1–3) (Table 5).

3.5. Estimated cost per protocol

Protocols that do not require any electrical equipment (protocols 1 and 5) were the most economical ones (Table 7). Among protocols that would need one or more pieces of sophisticated equipment for sample processing, protocol 3 was the most expensive one due to the cost of the centrifuge plate carrier. For laboratories with readily available equipment infrastructure, protocol 4 would be the least expensive procedure regardless of the number of samples to be processed, followed by protocol 5. The cost of protocols 1–3 and 6, which use multi-well plates for sample elution, varied depending on the number of samples being eluted. Overall, protocols 1, 5 and 6 would be easy and less costly to implement in any laboratory currently performing serology, with protocol 6 being the most advantageous in terms of sample volume recovery and effective liquid extraction.

4. Discussion

In the near future, DBS may become, together with oral fluid specimens, a practical specimen for the serology of rash illnesses, and it would be reasonable to think that several tests would be performed on a patient's DBS for differential diagnosis (Cubel et al., 1996; de Oliveira et al., 1999, 2000; Helfand et al., 2001). The goal of this paper was to provide an overview and a comparison of methods that have been used over the years to extract serum from DBS for the serodiagnosis of infectious diseases, and in particular for measles. Although our comparison was limited by the type and number of DBS samples used and by the use of a specific measles commercial enzyme immunoassay, our findings may serve as a guide on currently available protocols for DBS elution.

Our data suggest that any protocol based on active elution, i.e., protocols 3–6, would work well for the extraction of antibody for IgM detection in an indirect format EIA. In contrast, statistical differences were observed in eluates obtained by passive elution, i.e. protocols 1 and 2. Although, the optical density values obtained in the latter eluates were lower than in serum in the tested IgM positive sample, this did not lead to a result

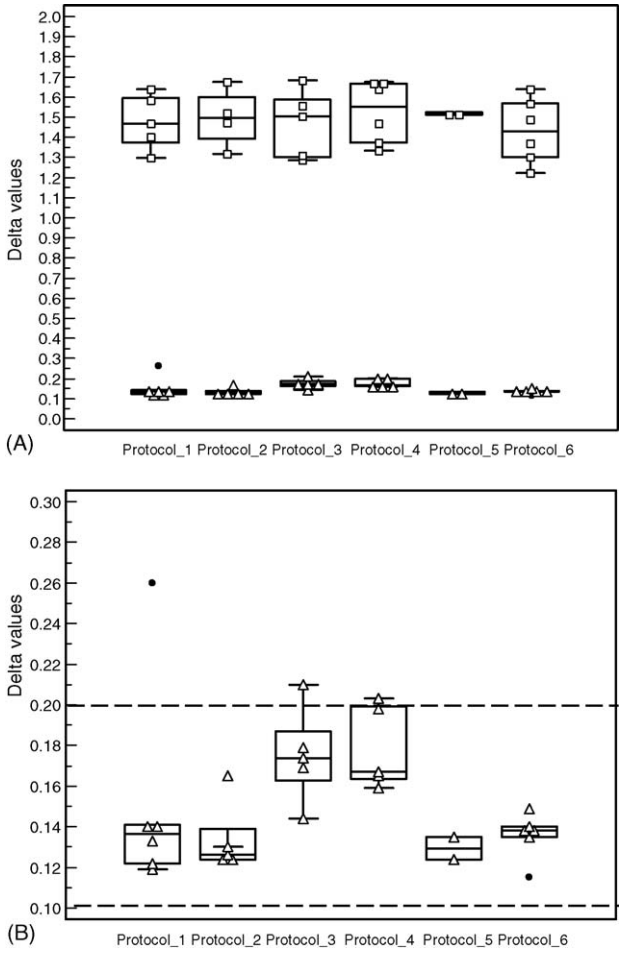

Fig. 3. Detection of antibody by the anti-measles virus IgG Dade Behring EIA after elution of an IgG positive and an IgG equivocal DBS samples by six different extraction protocols. Delta value is the optical density value of the positive well minus the optical density value of the negative well. Assay cut-off value for a positive sample is a delta value of 0.200. Assay cut-off value for a negative sample is a delta value of 0.100. A sample with a Delta value that falls in the interval 0.100–0.200 is considered equivocal. *Y*-axis minimum values in panel B is 0.100. Square markers: IgG positive DBS sample. Triangle markers: IgG equivocal DBS sample. Round black marker is a far out value.

misclassification. Unfortunately, an IgM positive sample with antibody reactivity close to the cut-off values was not available to study the effect of the observed statistical difference on a putative change of result classification, i.e., from positive to equivocal or negative. Interestingly, a passive elution protocol has been used in at least one evaluation study of DBS as alternative sample for serology that demonstrated an excellent correlation with serum by using a capture format EIA (Helfand et al., 2001). This could be explained by the fact that capture EIAs are designed to concentrate the target antibody resulting in higher detection sensitivity compared to indirect EIA. As for IgG detection, any of the protocols are likely to be useful for antibody elution. Unfortunately, differences among protocols detected with the IgG equivocal sample could not be further studied with a parallel comparison with serum. Hence, taking into account the EIA formats used for antibody detection, it is not surprising the good concordance observed between DBS and serum in performance evaluation studies despite using different DBS elution methods (Chakravarti et al., 2003; De Swart et al., 2001; el Mubarak et al., 2004; Helfand et al., 2001; Riddell et al., 2002, 2003).

On the other hand, differences among protocols were mainly found at the logistic and costing level. Protocol 6 was found to be the most advantageous procedure for outbreak situations, because it features a rapid removal method with recovery of ample specimen. In less than 8 h, ten DBS specimens received in the laboratory from remote outbreak areas can be eluted by protocol 6 and assayed by the Dade Behring IgM EIA. Furthermore, implementation of protocol 6 in serological laboratories should be seamless. Alternatively, protocols that employ 96-welled plates would work best for elution of large number of samples.

Limitations of this evaluation include the use of laboratory prepared DBS instead of clinical samples, which were not available at the time of this research. Also, testing of a low positive IgM or an indeterminate antibody sample - not available at the time – would have been beneficial to show differences in antibody detection at IgM levels close to the cut-off. Therefore, further evaluation would be needed using field specimens to verify that the presented observations hold true for clinical samples.

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