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Parameters of immunoglobulin extraction from dried blood spot cards and immunoassays for detection of antibody response to pathogens including the novel SARS-CoV-2

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ARTICLE INFO

Keywords:

Dried blood spots
Antibodies
Immune response
Antigens
Immunoassays

ABSTRACT

Dried blood spots (DBS) are routinely used in screening newborns for treatable disorders. Immunoglobulin extraction from DBS, serum or other biological fluids loaded on filter paper cards could represent a valuable method of specimen preservation in monitoring immune response against pathogens as well as vaccination efficiency. In this study using different sources including serum, and monoclonal antibodies we established parameters for antibody extraction from the filter cards to assess antibody reactivity against *Helicobacter pylori*, measles virus (MV) and the novel coronavirus SARS-CoV-2 antigens. We demonstrated that DBS and dried undiluted serum result in completely preserved antibody activity for immunoassays, including in virus neutralization assays against MV. Extraction efficiency was determined by IgG concentration measurements. The plaque-reduction neutralization titer 50% of dried human serum spots remained stable after more than 10-day storage – 1:359 vs. 1:345 for the corresponding frozen sample. DBSs could be used to monitor immune response to bacterial and viral antigens following natural exposure or immunization. Mice immunized with recombinant spike protein receptor-binding domain of SARS-CoV-2 developed a strong antibody response by day 14 and reached titers above 1:64,000 on day 21 following the secondary boost immunization as measured on DBS samples in antigen-mediated ELISA. Variability in IgG concentration of eluted DBS could be influenced by factors involved in sample application, extraction process and sample characteristics. Adjustment of antibody specific activity to the eluted IgG concentration can increase accuracy of the result interpretation, including in SARS-CoV-2 serological diagnostics.

1. Introduction

Dried blood spot (DBS) samples were first introduced in modern laboratory diagnostics by Guthrie for newborn screening (NBS) of inborn errors of metabolism (Guthrie and Susi, 1963). Recently the US Recommended Uniform Screening Panel has been expanded to include 35 primary and 26 secondary conditions every newborn in the US should be screened for (Advisory Committees on Heritable Disorders in Newborns and Children, 2021). Test on DBS specimens include not only inherited metabolic disorders, endocrinopathies, and hemoglobinopathies but also immunodeficiencies such as SCID (van der Burg et al., 2019). Blood samples from newborns are usually collected by heel-prick and directly deposited onto the half-inch filter paper circles on DBS

cards such as widely used Whatman 903 cards (Whatman, UK). In addition, application of the filter paper cards is not limited to the newborn screening purposes but also for adult blood testing. Sample collection and storage have many advantages over conventional venous blood collection including the potential for self-collection, reduced phlebotomist effort and shipment by regular mail (Lim, 2018). Relatively small volumes (approximately 50 μ l) of serum or other non-blood specimens could also be safely collected and transported for analysis. Filter paper samples are stable for longer periods of time when stored refrigerated or frozen. A small, 3 mm diameter size punches from the main circles are sufficient for extraction and subsequent analysis. Depending on the analytes of interest, properly stored DBS card specimens could be subject to further retrospective analysis years after

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<https://doi.org/10.1016/j.jim.2021.112996>

Received 19 October 2020; Received in revised form 25 January 2021; Accepted 2 February 2021

Available online 11 February 2021

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collection. Enzyme activity is well preserved on the DBS cards and the extracted soluble fraction can be subjected to multiplexed enzymatic analyses, such as screening for lysosomal storage disorders (Li et al., 2004). Besides the biochemical marker measurement, DBS could be used in toxicology marker probing, drug metabolite analysis and detection of DNA or RNA from different pathogens (Velghe et al., 2019; Ambach et al., 2019). An assay for detection of HIV, hepatitis C infection and malaria parasites on DBS samples has also been previously reported (Rottinghaus et al., 2013; Prinsen et al., 2020; Bereczky et al., 2005). Antibodies are stable in a dried form and extracts from DBS could be a useful tool in serological analysis of infectious and parasitic diseases (Baidjoe et al., 2013). DBS cards are valuable specimens for monitoring of transplacentally transferred maternal IgG that confers the passive immune protection against infections in the first months of life in newborns. Recently, this approach was also applied in predicting the protective immunity coverage in infants and optimal time for immunization with live measles, mumps, rubella (MMR) vaccine (Cilleruelo et al., 2019).

The current pandemic with the novel coronavirus SARS-CoV-2 emerged at the end of 2019 and has posed enormous challenges to healthcare systems globally (Malik et al., 2020; Lippi et al., 2020). Development of diagnostic tests was urgently demanded and included molecular diagnosis of the pathogen, serological tests and clinical biochemistry monitoring of infection (Bohn et al., 2020). The most common serological assays for retrospective confirmation of SARS-CoV-2 infection are ELISA and lateral flow rapid tests (Tuailon et al., 2020). Virus neutralization (VN) is the “gold standard” assay for detection of protective immunity against virus pathogens including SARS-CoV-2 (Haralambieva et al., 2008; Vaidya et al., 2010; Nie et al., 2020; Loefelholz and Tang, 2020). DBS-based serological assays with their simplified protocol of collection, transport, extraction and disposal could have major advantages in evaluation and tracking of population immunity in the ongoing pandemic. Initial data using ELISA and VN showed that infected individuals did not develop high antibody titers against SARS-CoV-2 antigens. VN titers reached maximum levels during the convalescent period corresponding to dilutions of 1:160–1:320 in microneutralization or plaque-reduction neutralization 50% test (PRNT₅₀) (Zhang et al., 2020; Haveri et al., 2020; Okba et al., 2020; Amanat et al., 2020). ELISA and VN antibody titers correlated with the severity of infection and could be close to the detection threshold in patients with mild disease: dilutions as low as 1:4 in VN and < 1:100 in antigen mediated ELISA against nucleoprotein (N) or spike protein (S) antigens have been employed in order to detect immune response. These data support the potential but also possible caveats of serological diagnostics. Extraction efficiency and other factors such as hematocrit could further influence the DBS-based serological assay’s sensitivity (Lim, 2018). The lower dilutions for detection of serological response to SARS-CoV-2 would require DBS extraction protocols that result in corresponding to recommended dilutions used in commercial ELISA kits. DBS adapted VN testing could be even more problematic because of the needed starting dilutions of <1:10. Thus, parameters of immunoglobulin extraction from DBS, including the actual IgG concentration and antibody specific activity specific are necessary for optimization and development of an accurate SARS-CoV-2 laboratory diagnostic assay.

Here we present evaluation of extraction efficiency and applicability of DBS-adapted immunoassays for detection of immune response to pathogens using blood, serum samples and monoclonal antibodies (MAbs) against different bacterial and viral antigens, including the novel SARS-CoV-2 S protein.

2. Materials and methods

2.1. Cell lines, MAbs and sera

Vero cells (ATCC) were grown and used for propagation of measles virus (MV) vaccine derivative strains as described before (Iankov et al.,

2011). Hybridoma 16F4 producing MAb against *Helicobacter pylori* neutrophil-activating protein (NAP) was generated using full-length 6-Histidine (6-His-) tagged NAP antigen (Iankov et al., 2011). MAbs 23C8 and 27H10 were generated using synthetic peptide corresponding to amino acid sequence 97–119 of NAP (Iankov et al., 2012). Strongly neutralizing anti-measles H protein MAb 20H6 (Iankov et al., 2013) was generated by immunization of interferon type receptor knockout and human CD46 transgenic mice (Ifnarko-CD46Ge) with live attenuated MV strain (Mrkic et al., 1998). Hybridoma culture supernatants or protein G affinity column (Thermo Fisher Scientific, Waltham MA) purified MAbs with determined concentration were used in the experiments. Sera from immunized Balb/c mice and Ifnarko-CD46 were collected at different time points and stored frozen at –20 °C. Human AB (+) blood group pooled serum was purchased from Sigma (St. Louis, MO), aliquoted and stored frozen.

2.2. Antigens and (MV) strains

6-His-tagged NAP antigen from *H. pylori* strain 26695 was extracted from bacterial cell expression system and purified using Ni-NTA Fast Start kit (Qiagen, Germantown, MD) as described before (Iankov et al., 2011). *H. pylori* heat-shock protein A (HspA) was inserted into pET-28a expression plasmid (Novagen, Madison WI) using BamHI/NotI cloning sites and expressed in *E. coli* BL21 Star (DE3) cells according to the recommended protocol (Thermo Fisher Scientific). The 6-His-tagged HspA was extracted under denaturing conditions and purified on the Ni-NTA Fast Start columns (Qiagen). Concentration of purified protein was measured by the UV light absorbance at 280 nm. Purified recombinant protein corresponding to the receptor-binding domain (RBD) of SARS-CoV-2 spike protein and derived from human cells (ProSci, Fort Collins CO) was used as antigen for immunization of mice. Recombinant His-tagged S-protein RBD reacting with ACE2 receptor (SynoBiologicals, Wayne PA) was used in antigen-mediated ELISA. Attenuated MV strain expressing green-fluorescent protein (MV-GFP) and MV encoding secretory form of *H. pylori* NAP protein (MV-s-NAP) were grown on Vero cells and titrated as previously described (Iankov et al., 2011; Duprex et al., 2000).

2.3. Animal immunizations

Animal studies were approved by the institutional animal care and use committee (IACUC). Groups of 5–6-week-old female Balb/c mice were immunized on day 1 with 5 µg purified HspA antigen in phosphate-buffered saline (PBS) mixed 1:1 with aluminum hydroxide adjuvant by intraperitoneal (i.p.) route. The immunization was repeated on day 21 and 28 and serum was collected on day 35 for checking immune response. Two months later mice were bled for DBS sample and serum collection. Group of 5 female Ifnarko-CD46Ge mice (Mrkic et al., 1998) were immunized by a single i.p. injection of 10⁶ TCID₅₀ of MV-s-NAP. DBS samples were collected on days 15 and 21 post immunization using Whatman 903 filter cards (Whatman, UK). The VN titers against measles were compared to samples collected on day 1 prior to immunization. In order to analyze immune response to purified SARS-CoV-2 spike antigen, 5 female Ifnarko-CD46Ge mice were injected i.p. on day 1 with 6 µg in 100 µl PBS recombinant S protein RBD (ProSci) formulated 1:1 with aluminum hydroxide (InvivoGen, San Diego, CA) to a final volume of 200 µl. Immunization was repeated on day 14 with 3 µg of the antigen administered through the i.p. route. DBS sampling was performed by tail bleeding on day 0 (prior to immunization), day 7, 14 and 21. Serum was collected on day 21 and stored frozen as described above.

2.4. DBS collection and storage

Blood from immunized mice was collected by tail vein bleeding and a single drop of blood was loaded onto the circled areas of the filter cards.

The cards were left to dry at room temperature for 30 min and subsequently refrigerated at 2–8 °C in plastic bags and used for the subsequent analysis. MAbs and sera were loaded on the filter paper and designated as purified antibody spot and serum spot filter paper samples respectively. The spots are made by adding 10 or 15 µl samples volume per circle.

2.5. DBS collection from patients with SARS-CoV-2 infection

Studies using human DBS samples have been approved by the Institutional Review Board (IRB), Mayo Clinic, Rochester MN. DBS specimens were collected from healthy individuals and convalescent patients with confirmed SARS-CoV-2 infection.

2.6. Filter paper antibody extraction

The circles with known volume of human serum, mouse serum, purified MAbs and hybridoma culture supernatants MAbs were cut out from the filter cards. Antibodies were extracted from whole in 0.2–0.5 ml PBS with 2% bovine serum albumin (BSA) and 0.05% Tween 20 (Sigma, St. Louis MO) following 2-h incubation on rocker platform at room temperature. DBS and serum from immunized mice were extracted from 3 mm filter card punches and incubated in 100–250 µl extraction buffer in 96-well plates as described above. For VN test antibodies were extracted in Opti-MEM medium (Thermo Fisher Scientific) with 2% BSA and antibiotics to prevent eventual bacterial contamination. Extracted samples were run on the assay within 24 h or stored frozen at –20 °C for subsequent analysis.

Human antibodies were extracted from DBS by incubation of single 3 mm punches in 150 µl PBS with 2% BSA and 0.05% Tween 20. Extraction was performed in round-bottom 96-well plates using a rotating platform for 2 h at 2–8 °C (Greiner Bio-One, ChromTec, Apple Valley, MN). Samples were diluted 1:2 in PBS, transferred to Sarstedt 12 × 75 mm polystyrene tubes (Thermo Fisher Scientific) and stored at 2–8 °C for subsequent analysis.

2.7. Antigen-mediated ELISA and capture ELISA

For antigen-mediated ELISA, 96-well plates were coated with 100 ng/well dissolved in 100 µl carbonate-bicarbonate buffer (CBB) pH = 9.6 of purified *H. pylori* NAP, HspA antigens or recombinant RBD of the novel SARS-CoV-2 S protein. Plates were incubated overnight at 2–8 °C, washed in PBS and blocked with 1% bovine serum albumin (Sigma) in PBS for 1 h at room temperature. Then plates were washed 3 times in PBS with 0.05% Tween 20 (PBS/T) and loaded with samples in dilutions (2-fold or 4-fold) in PBS/T with 2% BSA. Plates were washed 3 times in PBS/T and incubated with the corresponding goat anti-human IgG, A, M or goat anti-mouse IgG, A, M horseradish peroxidase (HRPO) conjugated secondary antibodies (Sigma) diluted 1:1000 in PBS/T with 2% BSA. The assay was run at two incubation time (30-min and 1-h incubation at room temperature) for both of the steps: sample incubation and secondary antibody incubation. Plates were washed 5 times in PBS/T and 100 µl TMB ELISA substrate (Bethyl laboratories) was added to the wells for 10–15 min at room temperature. The reaction was stopped using 100 µl 1 N HCl and the absorbance (OD_{450nm}) was measured on a Tecan 2000 Pro ELISA reader (Tecan, Switzerland). Antibody activity from dried filter paper extracts was compared to those of freshly diluted serum or MAbs. Wells coated with Ni-NTA extracts from pET-28 control plasmid transfected *E. coli* were used as controls in HspA and NAP antigen-mediated ELISA. For anti-S protein response analysis, control wells were incubated with CBB alone and blocked with BSA as described.

To assess the potential clinical application in detection of antibody response against SARS-CoV-2 S human DBS samples was tested in antigen-mediated ELISA against S-protein RBD antigen coated on 96-well ELISA plates as described above. Extracted samples collected from two seropositive patients and two seronegative individuals were

UV-inactivated (for 60 min), serially diluted in PBS/T with 2% BSA and run using 1:2 starting dilution and incubated for 30 min at room temperature on a rotating platform. Plates were washed 3 times in PBS/T and incubated with goat polyclonal anti-human IgG, A, M HRPO-conjugated secondary antibody (Sigma) diluted 1:1000 in PBS/T with 2% BSA for additional 30 min at room temperature. The assay was subsequently developed using TMB as a substrate and analyzed on a Tecan ELISA reader as described above.

The concentrations of mouse or human immunoglobulins in the extracted samples were measured by capture ELISA specific for mouse IgG or human lambda immunoglobulin chain (Bethyl Laboratories, Montgomery, TX) according to the manufacturer's protocol (Iankov et al., 2011).

2.8. VN test

Anti-measles human serum and MAb 20H6 VN titers were determined by plaque-reduction microneutralization assay and the plaque reduction neutralization titer 50% (PRNT₅₀) was calculated according to Karber's formula as previously described (Haralambieva et al., 2008). Briefly, the filter paper extracts were serially diluted in Opti-MEM, 2% BSA in 96-well plates (Corning, Corning NY). MV strain MV-GFP encoding green fluorescent protein (Duprex et al., 2000) was diluted in Opti-MEM and 250 tissue-culture infectious doses 50% (TCID₅₀) in 100 µl per well (1:1 with the antibody dilution) were added and incubated at 37 °C for 1 h. Then 100 µl of the mix were transferred onto Vero cells grown in 96-well plates and incubated for 72 h. GFP positive syncytia per well were counted on a fluorescent microscope and PRNT₅₀ titers were determined.

3. Results

3.1. Antibodies extracted from dried filter paper spots retained strong antigen-binding activity

The aim of these experiments was to determine extraction efficiency and specific activity of MAbs extracted from filter cards. Undiluted hybridoma supernatants of MAb 16F4 and 23C8 were loaded onto the filter cards and the corresponding whole circles containing 10 or 15 µl were extracted in 200, 300 or 500 µl PBS/T with 2% BSA. The dilution factor was calculated based on the volume loaded on the filter paper and extracted in the elution buffer volume. The same volume of frozen-stored original supernatants was diluted in the extraction buffer to the corresponding dilutions and used as controls. Samples were run in 4-fold dilutions up to 1:102,400 in duplicates in antigen-mediated ELISA against *H. pylori* NAP. Data showed that antibodies from undiluted supernatants containing 10% fetal bovine serum (FBS) were efficiently extracted and MAb activity was completely preserved as compared to control hybridoma supernatants (Fig 1A,B). The recovery of MAb specific activity at the different dilutions is shown in Fig 1C. In contrast, purified MAb 27H10 loaded in different dilutions and extracted from filter cards showed significant reduction in activity at the higher antibody dilutions (Fig. 1D).

3.2. VN activity of dried spot-extracted antibodies

VN is the most relevant reaction to demonstrate protective humoral immunity against virus infections. For VN testing, the immunoglobulin elution protocol was modified using Opti-MEM without Tween 20, in order conditions to be compatible with virus incubation and subsequent live cell culture. Filter papers were incubated in Opti-MEM without detergent. When the assay was performed using the purified MV hemagglutinin-reacting MAb 20H6 extracted from DBS, we observed that MAb 20H6 significantly lost its neutralization capacity after extraction from filter cards stored for 24 or 96 h at refrigerating conditions. Titer was reduced >5-8-fold to 1:1543 for the 24-h and 1:972 for

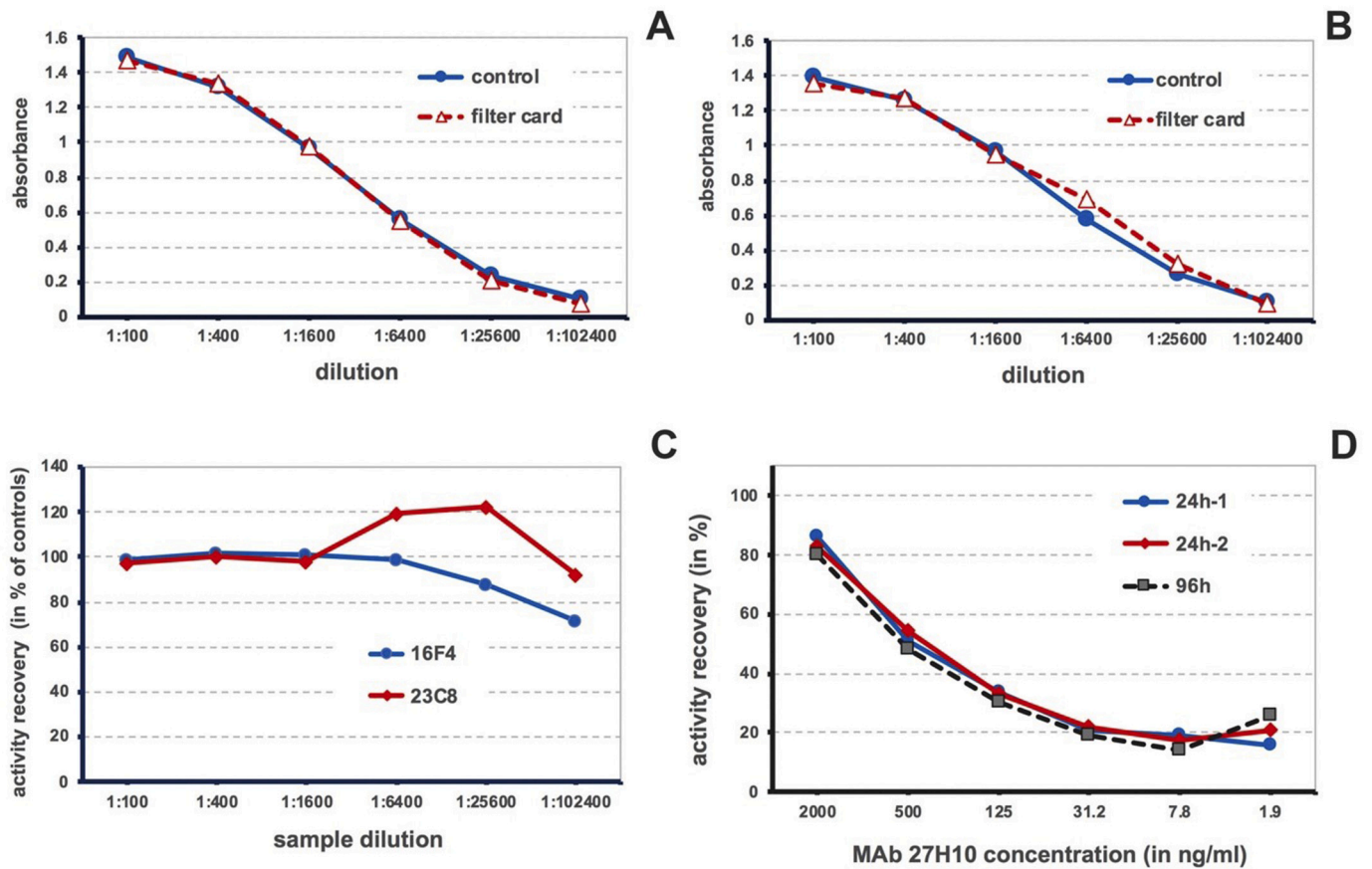


Fig. 1. Filter paper spots titration of NAP-specific MAb 16F4 (A) and 23C8 (B) supernatants in antigen-mediated ELISA against purified NAP. Recovery rate of MAb activity as compared to the control supernatants at different dilutions is shown in C. Purified MAb 27H10 had significantly decreased NAP-specific activity after loading at increased dilutions on the filter cards and subsequent extraction plates (D). Data for MAb 27H10 are presented as theoretically expected concentration in extracted buffer. Diluted samples were run in duplicates in 96-well ELISA plates.

the 96-h sample as compared to the control frozen MAb 20H6 PRNT₅₀ titer of 1:8396 (Fig. 2A). In contrast, neutralizing anti-measles activity of the human pooled AB(+) blood group serum was unaffected after spotting on the filter paper and stored for >10 days (Fig. 2B,C).

Titration experiments with different MV-GFP virus stocks determined the PRNT₅₀ titer of this serum lot to be between 1:155–1:371. Human serum samples of 10 µl loaded onto the card were extracted at two different dilutions - 1:20 and 1:50 in Opti-MEM. VN testing was

performed from these starting dilutions and titers were compared to corresponding dilutions of frozen serum in the same 96-well plates: 24-h, 96-h and 11-day storage of the cards did not significantly reduce serum specific activity vs. frozen serum. The titer following 1:50 extraction for example, was 1:281 for the 24-h and 1:359 for the 11-day stored samples respectively vs. a titer of 1:345 for the control serum. All samples, independently of the assay conditions had PRNT₅₀ above 1:120 that is considered protective for humans (Haralambieva et al., 2008).

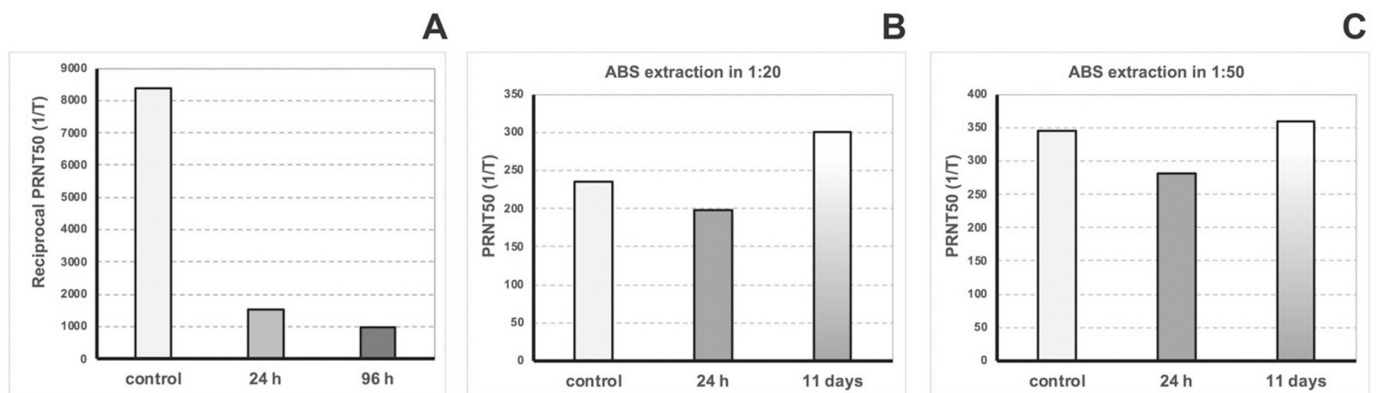


Fig. 2. VN test of high-grade purity MAB 20H6 (with defined starting concentration) against MV H protein extracted from DBS at different storage times before extraction (A). Titer in PRNT₅₀ test for anti-measles neutralization activity of human AB(+) blood group serum extracted in different Opti-MEM volumes corresponding to 1:20 or 1:50 dilutions (B,C). The titer was determined using a known amount of antibodies (10 µl serum transferred onto filter cards) and compared to that of freshly diluted serum. The PRNT₅₀ titer was calculated using Karber's formula and dilutions run in duplicates in 96-well cell-culture plates. Recovery rate determined by capture ELISA for human IgG-lambda was approximately 95% as compared to the frozen control serum.

Recovery of human IgG from DBS (>2-week storage and 30-min extraction) corresponded to 1.56 mg/ml serum concentration and that was comparable to 1.64 mg/ml of the control frozen AB(+) serum as measured by the human IgG-lambda quantitative ELISA. This data demonstrated that human serum specimens could be stored on filter cards and used subsequently for VN testing against live viruses without significant loss of the neutralization activity.

In order to address the question about the mechanism of monoclonal antibody activity decline following storage on filter paper, extracted MAb samples were analyzed for mouse IgG concentration compared to that of the original stocks. The results demonstrated that the reduced anti-MV titer of MAb 20H6 was due to inefficient extraction rather than impact on the immunoglobulin activity. IgG concentration was 8-fold lower in the extracted 10 μ l dried sample with recovery rate calculated at 12.57% (Fig. 3). In contrast, MAbs from supernatants had approximately 100% recovery rate for the mouse IgG. This data indicated that elution from dried filter paper spots of purified or diluted antibodies is significantly affected in the absence of the protective effect of high total protein concentrations.

DBS-derived immunoglobulins from MV-s-NAP-immunized mice were extracted after prolonged storage refrigerated for more than 60 days. The animals reached protective anti-measles average titers of 1:270 (range 1:176–1:384) by day 15 (Fig. 4A).

The specific immunoglobulin levels significantly increased to 1:1160 PRNT₅₀ (range 1:566–1:2820) on day 21 post immunization. When 3 mm DBS punches extracted in 100 μ l Opti-MEM the starting dilution was considered 1:100 (one 3 mm punch disk in 100 μ l) before mixing with the MV-GFP. Extraction efficiency was determined by mouse IgG-specific ELISA (Fig. 4B) and showed immunoglobulin amount corresponding to the IgG concentration in 1–1.5 μ l serum. A potential problem would be setting the assay with starting dilutions of >1:50. Not counting MV infection (syncytia number per well) at lower dilutions in the titration formula could impair the assay accuracy by calculation of artificially higher titers for control non-immune sera used to establish the VN cut-offs. Applying such as cut-offs could subsequently miss the MV neutralization effect or calculate lower relative titers for true positive MV immune sera. The alternative way to present data analysis is to calculate serum neutralization capacity as the absolute IgG amount

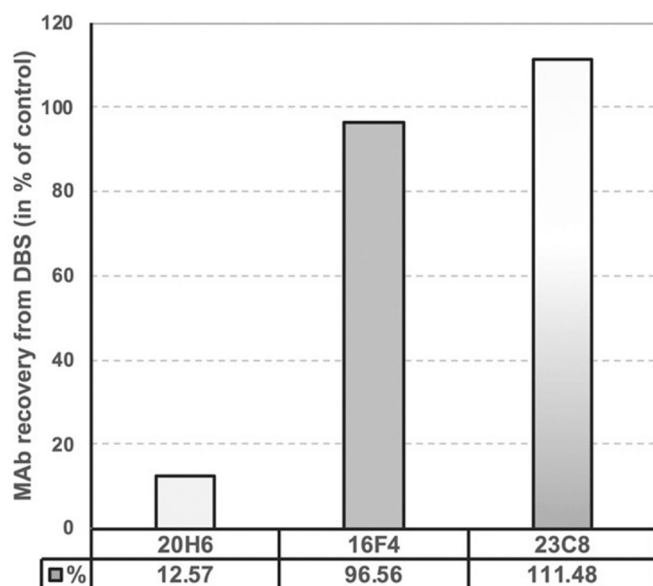


Fig. 3. Recovery rate of protein G affinity chromatography purified MAb 20H6 (IgG2a against MV) and two anti-NAP IgG1 MAbs 16F4 and 23C8 (supernatants with 10% FBS). Concentration of extracted MAbs was measured by ELISA run in duplicates in 4-fold dilutions. Recovery rate is presented as % of the control purified MAb or supernatants used for the filter paper samples.

adjusted to the concentration of extracted immunoglobulin fraction that is required for 50% reduction (IgG₅₀) of the virus infection (Fig. 4C). According to these data the minimum concentration of the extracted IgG for the assay should be approximately 90–100 μ g/ml. That concentration would allow detection antibody neutralization activity in sera with borderline protective titers in the range of 1:120 PRNT₅₀ considered protective against measles (Haralambieva et al., 2008).

3.3. DBS for detection of immune response against bacterial antigens

Different immunoassays, including bacterial agglutination reaction, ELISA and immunoblot against multiple or single antigens can demonstrate protective immunity against bacterial pathogens and their toxins or virulence factors. Balb/c mice were immunized with repeated i.p. injections of adjuvant formulated *H. pylori* HspA antigen. HspA is bacterial chaperone and key virulence factor with dual function as a Ni²⁺ scavenging protein (Schauer et al., 2010). To compare the DBS sampling in detection of anti-HspA immunity, a small volume of 2.5 μ l freshly collected blood and serum from 5 animals were transferred onto blood cards, dried and analyzed after storage for 72 h. After more than three months following primary immunization, mice maintained significant antibody response against HspA as measured by antigen-mediated ELISA. There was no significant difference in absorbance at 1:800 dilution for both dried blood and serum vs. freshly diluted corresponding serum (Fig. 5A). The measurement of extracted mouse IgG from DBS or dried serum spots showed the differences in IgG concentrations in the individual samples (Fig. 5B). These variabilities could be explained by difference in sample loading spread, extraction efficiency and hematocrit between individual mice.

3.4. Monitoring immune response kinetics following immunization with recombinant SARS-CoV-2 spike protein

A group of five 12–13-week old Ifnarko-CD46Ge mice were immunized on days 1 and 14 with two repeated injections of RBD (amino acids 319–541) of the S protein formulated with aluminum hydroxide as an adjuvant. Serum and DBS samples collected on day 0 prior to immunization served as controls. Mice were serially bled for DBS collection on day 7, before the second immunization on day 14 and on day 21. The antibodies were extracted from 3 mm filter paper punches incubated in 200 μ l ELISA buffer as described above. Antigen-mediated ELISA assays were run in 4-fold dilutions. A single immunization with the S protein antigen mounted robust antibody response in the mice by day 14 that was additionally boosted after the second immunization as measured on day 21 (Fig. 6A): all animals had titers above 1:25,600 (Suppl. Figs. S1 and S2). As the next step, we compared titration curves at different dilutions for DBS collected at 14 and 21 day as compared to the corresponding frozen sera collected on day 21. There was no significant difference between DBS stored more than a week vs. frozen serum for monitoring response to SARS-CoV-2 antigens (Fig. 6B). The use of polyvalent secondary HRPO-conjugated antibody could detect the collective antibody response contributed by the three major immunoglobulin isotypes – IgG, IgM and IgA.

Two administrations of the purified S RBD fragment induced very high serum titers >1:64,000 as determined by the endpoint dilution with absorbance 4 x SD above the average absorbance of sera collected prior to vaccination (Table 1). As expected, measurement of extracted IgG showed variability between the individual animals and time points of sample collection (Table 2). These results suggest that IgG concentration measurement and adjustment to specific IgG activity could provide more accurate assessment of antibody response in DBS, especially for samples close to the threshold of detection by a particular immunoassay.

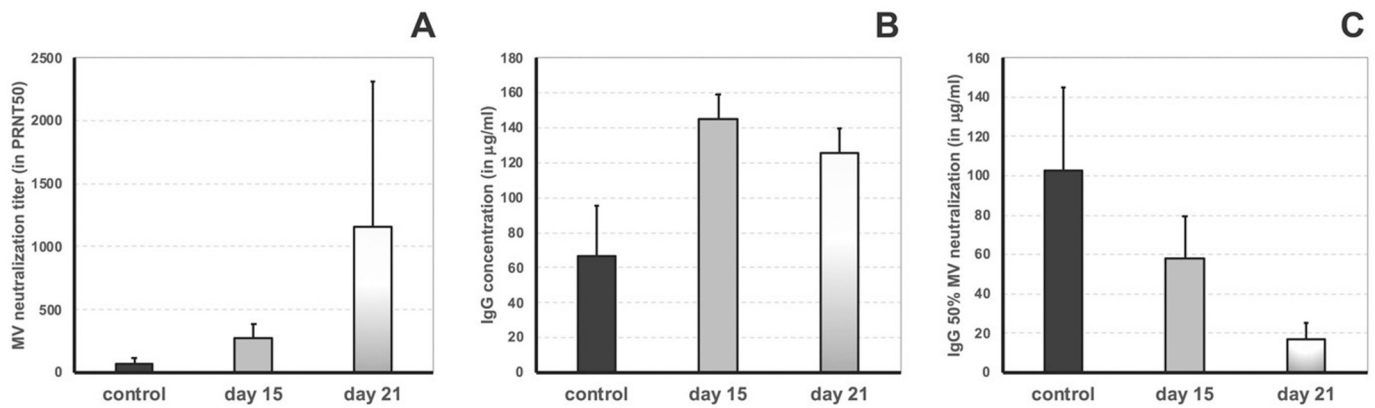


Fig. 4. Measles neutralization titer in MV-s-NAP-immunized Ifnarko-CD46Ge mice. PRNT₅₀ titers of antibodies extracted from DBS were determined on day 15 and 21 (A). Pre-immune DBS samples were used as controls. Individual IgG concentration of immunoglobulins extracted from DBS (3 mm punches incubated in 100 µl buffer) was determined by ELISA (B). The extracted IgG amount required for 50% reduction of MV infection was calculated based on the measured IgG concentrations (C). Data are presented as the average \pm SD bars for the samples (run in duplicates in 96-well plates) collected on the different days following immunization.

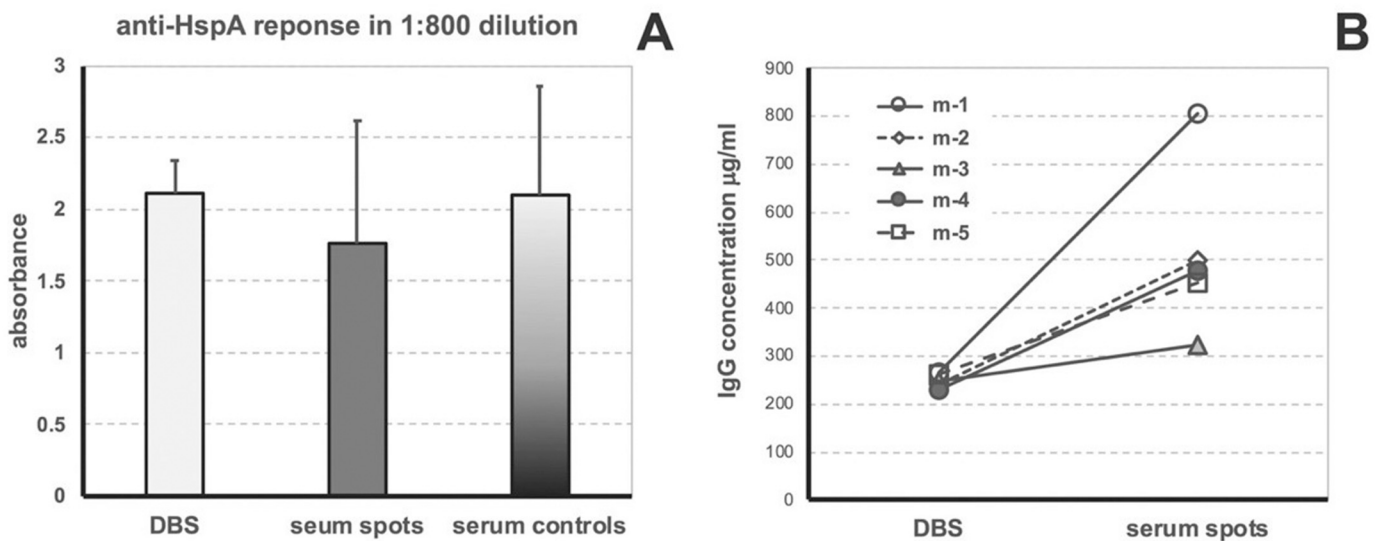


Fig. 5. HspA-specific antibody response in Balb/c mice. The anti-HspA ELISA was run using \sim 2.5 µl blood samples transferred to the cards and control serum dilutions adjusted to the theoretical hematocrit 50% (A). Results are presented as mean absorbance \pm SDs. Actual concentration of the extracted IgG paired for individual mouse - DBS vs. serum on the filter paper card (B). Each sample was run in duplicates in the ELISA plates.

3.5. Serological diagnosis of SARS-CoV-2 response in human DBS samples

In order to prove that the anti-SARS-CoV-2 humoral immune response could be detected in human DBS specimens, DBS samples from two individuals with confirmed recent SARS-CoV-2 infection (true positive) and two control samples (deidentified and labeled as A, B, C and D) were extracted and run in serial 4-fold dilutions in antigen-mediated ELISA against the S-protein RBD antigen. Data analysis demonstrated that a SARS-CoV-2-specific response was reliably detected in dilutions up to 1:32 in 3 mm DBS disks initially extracted in 150 µl sample buffer (Fig. 7). Absorbance reading at 1:2 dilution for SARS-CoV-2 convalescent samples was 1.076 and 1.411 (samples C and D) vs. 0.251 and 0.192 for controls (samples A and B) respectively. The collective antibody response was demonstrated using polyvalent human IgA, IgA and IgM specific secondary antibody. Concentration of the human IgG in the DBS extracts (heat-inactivated at 60 °C for 1 h) was measured by human immunoglobulin lambda chain specific ELISA and was in a range between 20.384 and 30.727 µg/ml.

4. Discussion

The ongoing SARS-CoV-2 pandemic demands development of rapid diagnostic assays for detection of active infection and sensitive serological tests for assessment of past exposure and seroconversion (Tang et al., 2020). The convenience of DBS specimen collection, transport and testing can minimize the risk of exposure for both patients and health care professionals.

In this study we aimed to determine the extraction parameters and reliability of immunoassays performed on blood, serum and purified antibodies spotted and dried on filter paper. A simple step of extraction directly in the ELISA incubation buffer worked very well for both DBS and dried serum samples. Because of the variability in hematocrit and serum IgG concentration, initial experiments on extraction efficiency and recovery of specific activity were run in comparison with well-characterized MAbs. Reaction in antigen-mediated ELISA of MAbs extracted from the filter spots was approximately equivalent to that of the control supernatants. Purified MAbs loaded on the filter paper however, showed significant reduction of reactivity, which we determined to be due to inefficient immunoglobulin extraction rather than

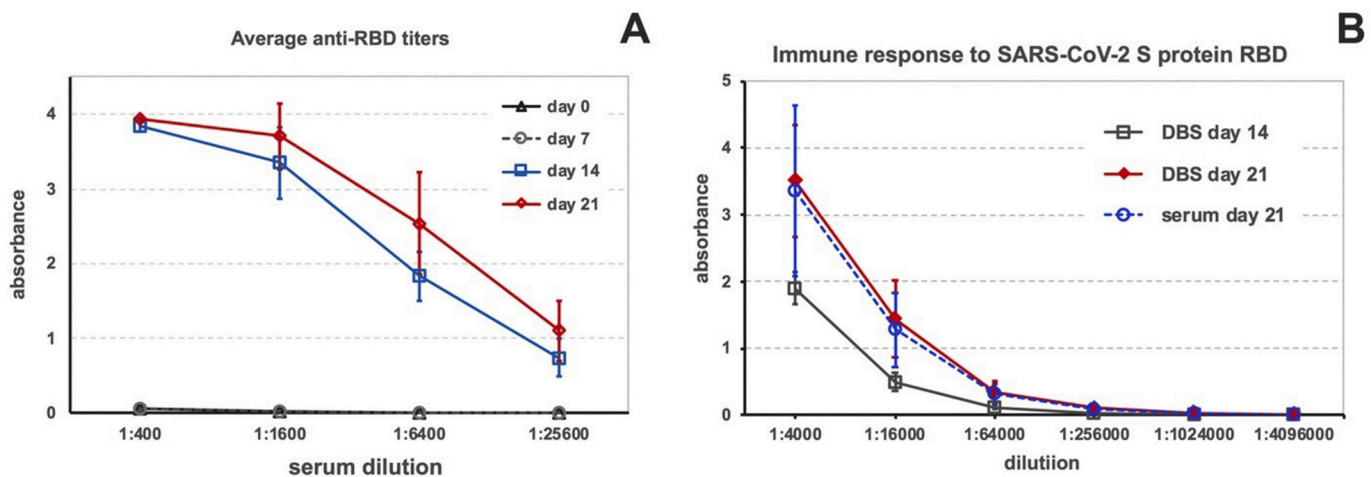


Fig. 6. Immune response to of SARS-CoV-2 recombinant S protein RBD antigen in mice. Titration in antigen-mediated ELISA of 4-fold diluted antibodies extracted from DBS at different study time points (A). Extended titration of the DBS samples collected on day 14 and day 21 compared to serum samples collected on day 21 as measured by antigen-mediated ELISA against spike RBD recombinant protein (B). Extracted samples were run in duplicates and results are presented as mean \pm SD (error bars) absorbance for the corresponding dilutions.

Table 1

Anti-S protein RBD titers in Ifnarko-CD46Ge mice following two i.p. immunizations on day 1 and 14 with purified SARS-CoV-2 RBD antigen. ND – not detectable.

	m-1	m-2	m-3	m-4	m-5
day 0	ND	ND	ND	ND	ND
day 7	<1:400	<1:400	<1:400	<1:400	<1:400
day 14	1:256000	1:64000	1:256000	1:64000	1:256000
day 21	1:1024000	1:1024000	1:1024000	1:64000	1:256000

Table 2

Total extracted IgG (in $\mu\text{g}/\text{ml}$) from DBS of the S protein RBD-immunized Ifnarko-CD46Ge mice as measured by ELISA. Range: 54.32–347.84; Mean: 188.92 ± 86.95 per dried spot.

	m-1	m-2	m-3	m-4	m-5
day 0	142.40	172.72	119.68	105.52	169.04
day 7	172.48	56.88	123.44	54.32	87.60
day 14	332.32	241.92	317.52	255.44	232.40
day 21	219.60	279.52	347.84	164.80	212.96

structural changes of the antibodies in the process. These data suggest that stability and extraction efficiency depend on the total protein concentration of the loaded sample. This could explain the protective effect of the 10% FBS on supernatant MAb activity. The protective effect of high protein concentrations should be considered when biological specimens with low protein content (eg. bronchial lavage, cerebrospinal fluid, saliva) are analyzed (filter paper sample advantages and different factor influence on analysis are summarized in [suppl. Table 1](#)). These types of samples are valuable sources for monitoring serological response during the SARS-CoV-2 pandemic ([Sullivan et al., 2020](#); [Ranadad et al., 2020](#)) and theoretically could be loaded on filter paper similar to the DBS collection. In this case, we would recommend the use of a high protein content buffer system (10% BSA, FBS, lactalbumin etc.) premixed 1:1 with the sample before spotting it on the filter paper. Filter paper sampling for serology analysis does not have to be limited to whole blood and serum. Introduction of dried filter paper sampling to test immunoreactivity of different biological fluids and cell culture conditions can further advance sample transportation storage and serological diagnostics. This approach should be explored also in an antigen-detection variant for rapid diagnostics of respiratory pathogens ([Hirotzu et al., 2020](#); [Porte et al., 2020](#)) using a protocol adapted for

extraction from filter paper samples.

DBS and serum spots analysis of the immune response to *H. pylori* antigens in mice showed no significant difference between extracted antibodies and control frozen serum samples from individual animals, indicating that the test is reliable and reproducible. An important conclusion was that variable factors, like hematocrit and serum IgG concentration in different samples could influence the titer calculations. Our data show that DBS and dried serum sampling may also be reliably employed for detection of the anti-SARS-CoV-2 antibody response in convalescent patients. Validation of the DBS-based immunoassays following the post-infection immune response time-kinetics in large group of patients could provide an enormous advantage as accessible means to assess immune response retrospectively but also to assess of vaccination efficacy. However, a key element for determination of ELISA's cut-off and interpretation of the results is the amount of immunoglobulin that can be isolated from the DBS or serum spots. We determined both the extraction efficiency and antibody specific activity by extracting immunoglobulins with known concentration and volume from large filter paper disks. ELISA-based methods for SARS-CoV-2 serology require sample dilutions of 1:100 or less ([Zhang et al., 2020](#); [Kohmer et al., 2020](#); [Van Elslande et al., 2020](#)). We calculated that IgG extracted from 3 mm punches corresponded to 1.5 to 3.5 μl blood. The assay run in 96-well plates requires sample volume of 50–100 μl . Thus, extraction of the 3 mm disks in larger than 100 μl volume would result in a suboptimal dilution factor that subsequently requires modification of cut-off values increasing the risk of high false-positive or false-negative rates. This could be of major importance in serological diagnostics following longer periods of time post-infection, immune response in individuals with mild infection forms and in monitoring the immunization efficacy. Based on our results, we suggest extraction of 6 mm or larger punches in buffer volumes that would correspond to 1:50 or lower dilutions of blood or serum that are required for other immunoassays, including VN tests. The use of a poly-reactive secondary antibody recognizing the major immunoglobulin isotypes IgG, IgA and IgM provided the advantage to capture the total antibody response, thus increasing the assay sensitivity. Measurement of the IgG in the extracted samples is essential for accurate result interpretation and in order to contain the false positive and false negative rates. The immunoassays could be easily adapted to analyze IgM-mediated early response or IgA (as the principal mucosal defense antibodies) but specific activity should be calculated by measurement of concentration of the corresponding immunoglobulin isotype in DBS-extracted samples.

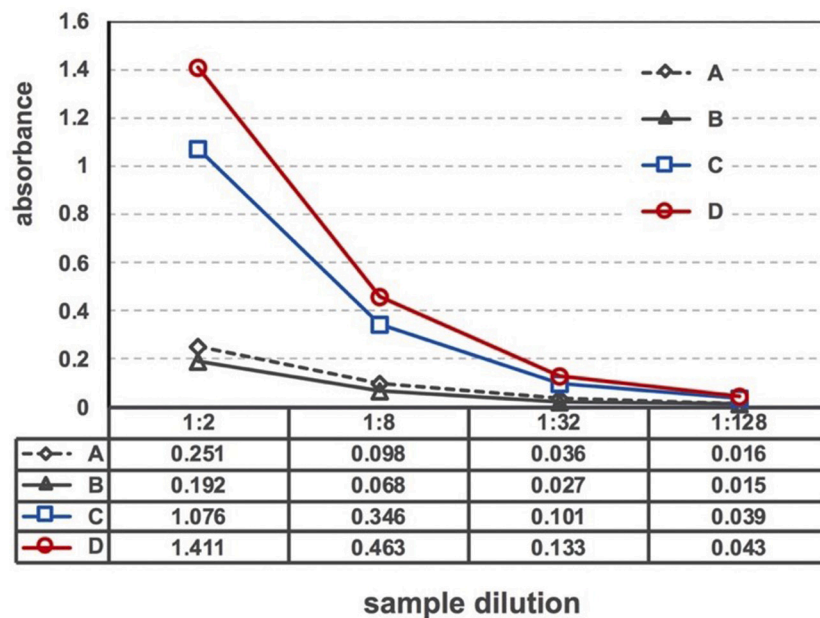


Fig. 7. Immune response to recombinant S protein RBD antigen in SARS-CoV-2-convalescent individuals. Samples were run in 4-fold dilutions from 1:2 to 1:128 in antigen-mediated ELISA with plates coated with 100 ng per well antigen. The absorbance (OD_{450nm}) reading for individual samples at different dilutions is presented in the table below the figure. Extracted IgG concentration as measured by human IgG-lambda ELISA (run in 2-fold dilutions) for individual samples was calculated: A = 20.384 μ g/ml, B = 20.570 μ g/ml, C = 30.727 μ g/ml and D = 21.322 μ g/ml respectively. The ELISA assay was run in duplicates in 96-well plates and mean concentration was calculated for each of the samples.

VN is considered as the gold standard test for measurement of protective immunity against viruses following vaccination or natural infection (Haralambieva et al., 2008; Whiteman et al., 2018). Potential caveats in VN performed on DBS specimens could be extraction conditions and smaller extraction volumes in order to run the assay at lower dilutions. Since VN is run by overlay onto cell monolayers, any detergent, including Tween 20 present in ELISA buffer, will be incompatible with the assay. Our results showed that extraction in reduced-protein tissue culture medium, such as Opti-MEM could provide the optimal conditions for subsequent VN analysis. Presence of 1–2% BSA could prevent potential non-specific absorption of the antibodies on the surface of the polystyrene plate well in the extraction process. Because the filter paper spots are not collected and stored in sterile environment we recommend the use of antibiotic-supplemented medium. MV was used as prototype virus pathogen to determine and optimize the VN using DBS and serum spots. Incubation of the filter paper punches in Opti-MEM at room temperature for 2 h was sufficient for immunoglobulin elution. We employed a known loaded volume of human AB(+) blood group serum and MV-neutralizing MAb 20H6 in order to determine the IgG recovery and specific activity. Specific activity of serum antibodies was not affected following storage for more than 10 days and showed no significant difference from control frozen serum samples. Neutralization activity of DBS tested during the course of immunization of mice with MV was preserved, indicating that prolonged sample storage had no significant negative impact on the assay results. However, the dilution factor is a very important parameter that can interfere with the calculation of the PRNT₅₀ titer against measles. Because the measles-protective titer for humans has been determined to be 1:120 (Haralambieva et al., 2008), starting dilutions of 1:20–1:40 should be recommended to avoid interpretation discrepancies at serum dilutions <1:120 (Ratnam et al., 1995). Hematocrit, immunoglobulin concentration and any inconsistency of the DBS could increase the variability. Our data showed that specific neutralization adjusted per μ g of extracted IgG would be the most accurate approach for the VN test interpretation. Detection of SARS-CoV-2 neutralizing antibodies in convalescent patients requires a dilution threshold that could start at 1:10 or even at 1:4 (Ko et al., 2020; Lu et al., 2020). If the specimen is DBS, the best approach would be to excise an entire circle from the filter cards and elute it in 200–500 μ l serum-free medium. Measurement of the extracted immunoglobulins and correction to the IgG concentration specific

activity in this case should be the method of choice for the most accurate titer calculation.

In conclusion, DBS and serum card spots can be valuable sources for testing immune response to bacterial and viral pathogens; this approach warrants further testing using different biological fluid specimens and can be expanded to detect IgM as an early infection marker and secretory IgA as the principal mucosal defense antibodies.

Declaration of Competing Interest

The authors have no competing interests to declare.

Acknowledgements

Manuscript was supported in part by National Institute of Health (NIH)/National Cancer Institute (NCI) grants R01 CA200507, R01 CA258239 and P50 116201 (EG).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jim.2021.112996>.

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