Video Article

A Protein Microarray Assay for Serological Determination of Antigen-specific Antibody Responses Following *Clostridium difficile* Infection

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Abstract

We provide a detailed overview of a novel high-throughput protein microarray assay for the determination of anti-*Clostridium difficile* antibody levels in human sera and in separate preparations of polyclonal intravenous immunoglobulin (IVIg). The protocol describes the methodological steps involved in sample preparation, printing of arrays, assay procedure, and data analysis. In addition, this protocol could be further developed to incorporate diverse clinical samples including plasma and cell culture supernatants. We show how protein microarray can be used to determine a combination of isotype (IgG, IgA, IgM), subclass (IgG1, IgG2, IgG3, IgG4, IgA1, IgA2), and strain-specific antibodies to highly purified whole *C. difficile* toxins A and B (toxinotype 0, strain VPI 10463, ribotype 087), toxin B from a *C. difficile* toxin-B-only expressing strain (CCUG 20309), a precursor form of a B fragment of binary toxin, pCDTb, ribotype-specific whole surface layer proteins (SLPs; 001, 002, 027), and control proteins (tetanus toxoid and *Candida albicans*). During the experiment, microarrays are probed with sera from individuals with *C. difficile* infection (CDI), individuals with cystic fibrosis (CF) without diarrhea, healthy controls (HC), and from individuals pre- and post-IVIg therapy for the treatment of CDI, combined immunodeficiency disorder, and chronic inflammatory demyelinating polyradiculopathy. We encounter significant differences in toxin neutralization efficacies and multi-isotype specific antibody levels between patient groups, commercial preparations of IVIg, and sera before and following IVIg administration. Also, there is a significant correlation between microarray and enzyme-linked immunosorbent assay (ELISA) for antitoxin IgG levels in serum samples. These results suggest that microarray could become a promising tool for profiling antibody responses to *C.difficile* antigens in vaccinated or infected humans. With further refinement of antigen panels and a reduction in production costs, we anticipate that microarray technology may help optimize and select the most clinically useful immunotherapies for *C. difficile* infection in a patient-specific manner.

Video Link

The video component of this article can be found at https://www.jove.com/video/57399/

Introduction

This protocol describes the development and validation of a novel and customized protein microarray assay for the detection and semi-quantification of bacterial strain and isotype-specific antibody responses to *C. difficile* antigens. We successfully use our *C. difficile*-specific microarray assay as a promising new tool for the compositional bioanalysis of specific antibody content in patient sera[1,2], preparations of IVIg[3], and identification of antibody specificities that correlate with poor outcomes in CDI[4]. We demonstrate how biobanked serum samples and commercial preparations of IVIg can be analyzed on microarray slides, allowing high-quality reproducible profiling of *C. difficile* pathogen-specific antibody responses in this assay.

Many healthy children and adults have detectable serum IgG and IgA antibodies to *C. difficile* toxins A and B[5,6]. These are thought to arise following transient exposure during infancy and following exposure to *C. difficile* in adulthood. For this reason, polyclonal IVIg has been used off-label to treat both recurrent and fulminant CDI[7,8,9]. However, its definitive role and mode of action remains unclear. Several studies have shown that the humoral immune response to *C. difficile* toxins plays a role in disease presentation and outcome. Specifically, asymptomatic patients show an increased serum anti-toxin A IgG concentration compared to patients who develop symptomatic disease[10]. A demonstrable association has been reported for median anti-toxin A IgG titers and 30-day all-cause mortality[11]. Several reports have also revealed an association with a protection against recurrence and antibody responses to toxin A, B, and several non-toxin antigens (Cwp66, Cwp84, FliC, FliD, and surface layer proteins (SLPs))[12,13,14,15]. These observations have spurred the development of the first passive immunotherapy drug targeting *C. difficile* toxin B (bezlotuxumab), which has recently been approved by the US Food and Drug Administration and the European Medicines Agency for the prevention of recurrent CDI[16]. Vaccination strategies using inactivated toxins or recombinant toxin fragments are also currently under development[17,18,19]. These new therapeutic approaches will undoubtedly stimulate the requirement for evaluating humoral immune responses to multiple antigens in large sample sizes.

Today, there is a notable lack of commercially available high-throughput assays capable of simultaneously assessing bacterial strain and isotype-specific antibody responses to *C. difficile* antigens. There is an unmet need to develop such assays to facilitate future research efforts and clinical applications. Protein microarrays are a method to immobilize large numbers of individually-purified proteins as a spatially organized array of spots onto a microscopic slide-based surface by using a robotic system, which can be either a contact[20] or a non-contact printing tool[21]. The spots may represent complex mixtures such as cell lysates, antibodies, tissue homogenates, endogenous or recombinant proteins or peptides, body fluids or drugs[22,23].

Protein microarray technology offers distinct advantages over standard in-house ELISA techniques, which have traditionally been used to assess anti-*C. difficile* antibody responses. These include an increased capacity for detecting a range of multi-isotype-specific antibodies against a more extensive panel of protein targets, reduced volume requirements for antigens, samples, and reagents, and an enhanced ability to incorporate a larger number of technical replicates, in addition to multiple internal quality control (QC) measures[1]. Microarrays are therefore more sensitive, accurate, and reproducible and have a greater dynamic range. These factors make microarrays a cheaper and potentially favorable alternative to ELISAs for the large-scale detection of known proteins. However, disadvantages of microarray technology result mainly from the large up-front costs associated with establishing a panel of highly purified antigens and setting up the technological platform.

Protein microarrays have been extensively used over the past two decades as a diagnostic and basic research tool in clinical applications. Specific applications include protein expression profiling, the study of enzyme-substrate relationships, biomarker screening, analysis of host-microbe interactions, and profiling antibody specificity[23,24,25,26,27,28]. Many new pathogen protein/antigen microarrays have been established, including malaria (Plasmodium)[29], HIV-1[30], influenza[31], severe acute respiratory syndrome (SARS)[32], viral hemorrhagic fever[33], herpesviruses[34], and tuberculosis[35].

The present protocol relates to the establishment of an easy operating *C. difficile* reactive antigen microarray assay, which enables accurate, precise, and specific quantification of multi-isotype and strain-specific antibody responses to *C. difficile* antigens in sera and polyclonal IVIg. Herein, we include representative results pertaining to an acceptable microarray assay performance when compared to ELISA as well as assay precision and reproducibility profiles. This assay could be further developed to profile other clinical samples and sets a new standard for research into the molecular basis of CDI.

Protocol

1. Preparing a Microarray Plate

1. Dilute *C. difficile* antigens with a printing buffer [PBS-Tween-trehalose (50 mM)] at the optimum concentration (which was predefined before running the patient sera): toxin A (200 µg/mL) and toxin B (100 µg/mL), pCDTb (200 µg/mL), and purified native whole SLPs derived from ribotypes 001, 002, and 027 (200 µg/mL).
   NOTE: Toxin B was obtained from a toxin B-expressing strain CCUG 20309 (90 µg/mL).
   1. Dilute the positive controls, lysates of *C. albicans*, and tetanus toxoid with the printing buffer at 100 µg/mL. Finally, dilute the purified human immunoglobulin matching the tested antibody isotype serially, starting at 50 µg/mL across 10 dilutions in the printing buffer to create a calibration curve.
2. Transfer 10 µL of the dilutions in the printing buffer into a 384-well plate.
3. Cover the plate with a plate seal and centrifuge at 300 x g for 5 min.

2. Printing Arrays with a Contact Robot

1. Heat the silicon pin using the hand-held gas burner 3x 2 s each and rinse in clean water 3x 3 s each.
2. Place the microarray plate into the loading cartridge of the arrayer.
3. Place the aminosilane slides on the slide tray.
   NOTE: The slide tray can hold 27 slides: three rows of nine slides. The slides are arranged in portrait orientation starting from the left side of the bottom row and the rest of the spaces are covered with blank slides to ensure that all the holes on the slide tray are covered.
   1. Press **OK** and check that a vacuum has been turned on and all the slides are secure. Leave the slides in the humid environment for at least 1 h before starting the print.
      NOTE: The aminosilane slides are provided in a sealed pouch with desiccant. Once opened, any unused slides should be returned immediately to the desiccator for storage up to the expiration date.
4. Set up the suitable printing programme to print *Clostridium difficile* antigens. Launch the TAS Application Suite and open the required print run parameters file from the 'My Gridding Runs' directory. Select *Clostridium difficile* file for printing all the *Clostridium difficile* antigens in quadruplicate.
5. Upon double-clicking on the MicroArray icon on the main window a three-tabbed window will appear called ‘MicroArraying parameters’. The three tabs are “Source,” “Target,” and “Options.” The “Source” tab shows details the number of samples used in the microarray plate. The “Target” tab shows details of how the slides are to be loaded, where the array should be printed on the slide and edit the slide layout (16 subarray formats). The “Options” tab shows details of the wash protocol and tool to be used *e.g.* wash after every completed sample. Clean the silicon pin between samples to avoid any cross contamination between different samples.
6. The programming options are located under Options>Run Preferences on the TAS Application Suite toolbar. There are 9 tabs to work through in this section and as you complete one tab move to the next by clicking on it. Clicking the “OK” the button will take you out of “Run Preferences.” In the “General” tab of the “Run preferences,” there are a number of check boxes available under this section relating to how the instrument will behave when a program is started. Check the “Prime bath before start of run” box, all three wash stations will undergo a short priming sequence prior to the run beginning. Check the “Wash at start of run” box, the pin tool will be washed before the first source visit. Check “Wash at end of run” box, the pin tool will be washed after the last source visit. The user can set the number of wash cycles. In

the “Climate” tab of the “Run preferences,” start the humidification. The setting we use are as follows: start rate 55%, run rate 55%, minimum humidity 55%, target humidity 60%. Do not start the run until the target humidity has been reached, otherwise the spots will be the wrong size and the library will rapidly evaporate.

7. In the menu bar of TAS, click the green go button. Start the run and periodically observe the arrayer during the print run to be certain everything is OK.
   Note: This enables the analysis of 15 samples in parallel on each slide as one subarray is used as a negative control. The design of subarrays is determined by the number of the printed proteins (antigens) and the number of the printed biological replicates.
   Note: After printing each sample, the head moves toward the washing baths to allow multiple dipping of the pin into two wash baths containing distilled water and 0.002% Tween 20 for 1.5 s in each bath, followed by rinsing the pin with ultra-pure water and drying the pin in the main wash station for 4 s.

3. Storage of the Arrays

1. Keep the printed slides stored overnight at room temperature in the desiccator.
   NOTE: The printed slides can be stored for up to one week.

4. Array Probing

1. Carefully place printed slides in a slide holder of 16 multi-well chambers format.
   NOTE: The chambers, having a depth of approximately 7.5 mm, provide a generous surface to volume ratio to facilitate mixing and washing steps.
2. Allow all reagents to warm to room temperature before use. Unless specified otherwise, perform all the following steps at room temperature.
3. Add 100 µL of filtered 5% bovine serum albumin tween (BSAT; use a 0.2 µm syringe filter) to each block, cover the slides, and incubate them with shaking for 1 h.
4. Wash the slides 5x 1 min each in 120 µL of phosphate buffered saline with Tween 20 (PBST; 0.1% Tween) per well with shaking. Do not let the slides dry out.
5. Thaw the serum samples on ice for 20 min and dilute each sample with a commercially available antibody diluent with background reducing component (see **Table of Materials**).
   1. Optimize the dilution of the serum samples according to the tested immunoglobulin as shown in **Table 2**.
6. Transfer 100 µL of the diluted serum samples to all blocks except one, which will be used as a negative control; to this block, add 100 µL of antibody diluent only. Incubate the slides with gentle agitation for 1 h.
7. Wash the slides 5x 3 min each in 120 µL of PBST (0.1% Tween) per well with shaking.
8. Add 100 µL of a biotinylated goat anti-human antibody diluted with antibody diluent.
   1. Optimize the dilution of the secondary antibody according to the tested immunoglobulin as described in **Table 2**. Cover the slides and incubate them with gentle shaking for 1 h.
9. Wash the slides 5x 3 min each in 120 µL of PBST per well with shaking.
10. Add 100 µL of streptavidin Cy5 to each block diluted at 1:2000 in 5% BSA. Cover the slides with foil to protect them from light whilst shaking for 15 min with gentle agitation.
11. Wash the slides 5x 1 min each in 120 µL of PBST per well with shaking, followed by 2 washes of 1 min each in PBS only with shaking.
12. Spin the slides for 5 min at 300 x g to dry them.
13. Keep the slides in a dark box for transport and storage at room temperature.
14. Proceed to scan the arrays immediately to ensure signal consistency after probing.
    NOTE: However, probed slides can be stored in the dark and scanned within one week from probing.

5. Scanning the Arrays

1. Switch on the laser scanner (see **Table of Materials**) 30 min before scanning to warm up the laser. Upload the scanner software and connect the computer to the scanner.
2. Place a slide with the printed side face up into the scanner until the play light turns solid green.
3. Press the **Settings** button and adjust the following settings when scanning the slides as follows: Scan Mode, Median; Acquisition, 635 Only; Acquisition Mode, Manual; Gain, 20; Power, Low; Slide Type, Unlabeled Slide; Focus, Auto Focus. Turn Automatic Scrolling off and set Barcode Reading to Pixel Size 10 and Scan 35.
4. Save the resultant images as 16-bit grayscale multiple image TIFF format. Press the **Import Grid** button and select the appropriate array list.
   NOTE: The array list describes the size and position of subarrays and the names of the printed substances associated with each feature-indicator.
   1. Align the grid to the spots on the slide.
5. Analyze the images by pressing the **Quantification process** button to measure the fluorescence signal intensities of each spot and save the results in a text format called GenePix Results (GPR).
   NOTE: The GPR file contains the localization and identification variables of the antigen targets on the array and also the median fluorescence intensity and the local background that represents the antibody binding signal values of each spot.

6. Data Analysis

1. Calculate the median for the replicates of each antigen after background subtraction using a free microarray analysis software called reverse phase protein array (RPPA) analyzer, a module within the R statistical language on CRAN[36].

2. Plot the standard curve and interpolate the signals of each antigen relative to the immunoglobulin standard curve using commercial scientific graphing and statistics software (see **Table of Materials**) to calculate the antibody levels.

Representative Results

Figure 1 illustrates a flowchart describing the major steps in the described protocol. **Figure 2** shows Spearman correlation tests demonstrating significant agreement between microarray and ELISA for IgG and IgA anti-toxin A and B levels in the patient test sera. **Figure 3** shows differential IgG and IgA antibody-class specific antibody responses to toxin A, toxin B, and binary toxin (pCDTb) in patients with CF without diarrhea, CDI patients with diarrhea, and in HC. **Figure 4** shows *C. difficile* antitoxin neutralizing antibody responses in the patient sera. **Figure 5** shows the immune reactivity (against *C. difficile* toxins and SLPs) and neutralizing effect (against *C. difficile* toxins) of IVIg. **Figure 6** shows the immune reactivity (against *C. difficile* toxins and SLPs) and neutralizing effect (against *C. difficile* toxins) of the patient sera pre- and post-IVIg administration. **Table 1** shows acceptable intra- and inter-assay coefficients of variability of microarray using test sera. **Table 2** illustrates a list of immunoglobulins used in this study with relevant concentrations of purified proteins as well as optimized dilutions for sera and secondary antibodies.

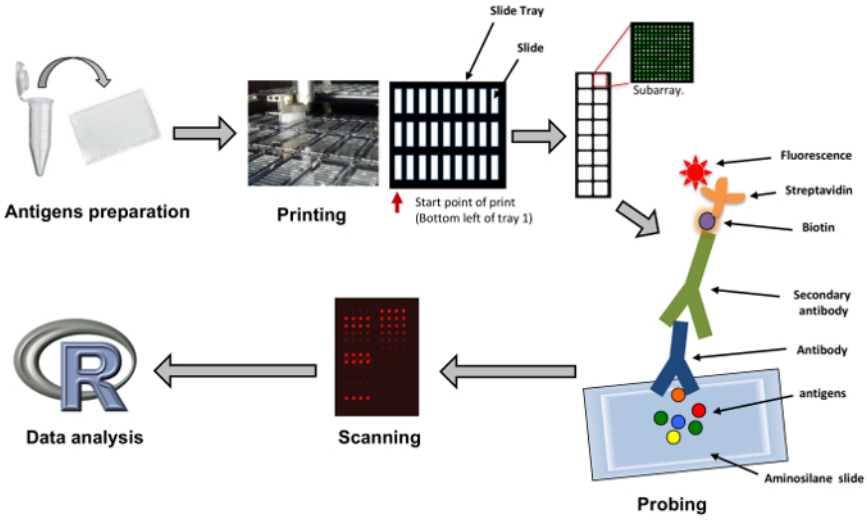

Figure 1: General overview of major steps involved in the microarray protocol. The first step is the preparation of the antigens and controls. The subsequent sample dilutions are transferred to a 384-plate in readiness for printing in quadruplicates onto the aminosilane slides. Following drying and blocking of slides, the arrays are incubated with patient sera. After further washing, the biotinylated anti-human immunoglobulin (Ig) of the specified isotype is added. After the final washing and drying steps, the slides are scanned and the resultant images processed with a microarray image analysis software. Please click here to view a larger version of this figure.

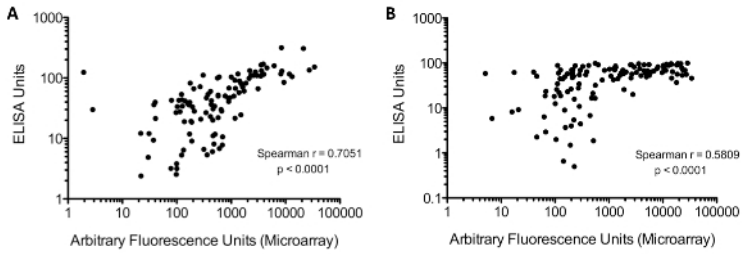

Figure 2: Correlation between microarray and ELISA results. The Spearman correlation coefficient was used to assess the level of agreement between the two platforms. When comparing the microarray performance with an in-house enzyme-linked immunosorbent assay (ELISA), **A**. a good correlation coefficient was observed for toxin A (r = 0.7051; P <0.0001), **B**. and a moderately good correlation for toxin B (r = 0.5809; P <0.0001). The full ELISA protocol has been detailed elsewhere[37]. This figure has been reprinted from Negm *et al.*[1] with permission. Please click here to view a larger version of this figure.

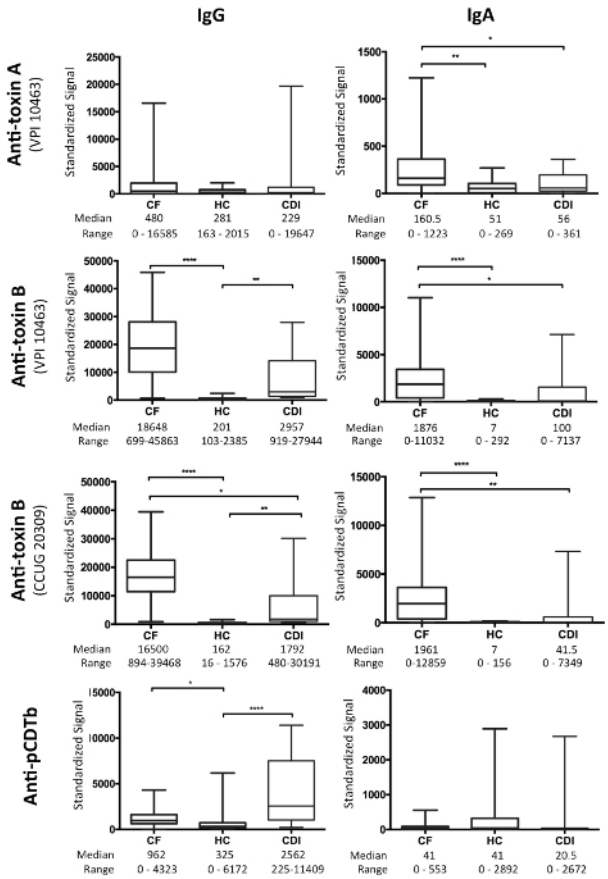

Figure 3: **Isotype-specific antibody responses to *Clostridium difficile* toxins**. This image sows the serum anti-toxin IgG and IgA responses (toxinotype 0, strain VPI 10463, ribotype 087; toxin A at 200 µg/mL, toxin B at 100 µg/mL), toxin B (*C. difficile* toxin B-producing strain CCUG 20309; toxin B at 90 µg/mL) and the precursor form of a B fragment of binary toxin, pCDTb (200 µg/mL), in patients with cystic fibrosis (CF) without diarrhea, patients with *C. difficile* infection (CDI) with diarrhea, and in healthy controls (HC). The serum dilution for IgG and IgA are 1:500 and 1:100, respectively. The differences between the groups were assessed using the Kruskal-Wallis test, followed by Dunn's post hoc test for multiple responses. Compared with the HC (n = 17) and the patients with symptomatic CDI (n = 16), the adult CF patients (n = 16) exhibited significantly higher levels of serum IgA anti-toxin A and B levels; $P \leq 0.05$. The same pattern prevailed for IgG, except that there was no difference in the anti-toxin A IgG levels between the groups. The box and whisker plots represent the median, range, and quartiles. *** $P \leq 0.0001$; ** $P \leq 0.01$; * $P \leq 0.05$. The standardized signals are normalized to the immunoglobulin standard curve. This figure has been reprinted from Monaghan *et al.*[2] with permission. Please click here to view a larger version of this figure.

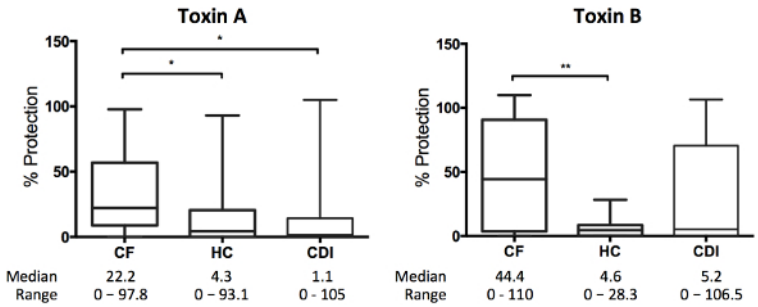

Figure 4: **Neutralizing antibody efficacies to *C. difficile* toxins A and B in patients' sera.** This figure shows the protective neutralizing antibody (NAb) responses to *C. difficile* toxins A and B [toxinotype 0, strain VPI 10463, ribotype 087, used at a 50% lethal dose (LD50)] in the sera (1:100 dilution; toxin A 2.5 ng/mL, toxin B 0.5 ng/mL) from the HC, the patients with CF without diarrhea, and the patients with CDI with diarrhea. The sera from CF patients exhibited significantly stronger protective anti-toxin NAb responses compared with the sera from the HC (toxins A and B) and from the patients with CDI (toxin A). The differences between the groups were assessed using the Kruskal-Wallis test, followed by Dunn's post hoc test for multiple responses. The box and whisker plots represent the median, range, and quartiles. ** $P \leq 0.01$; * $P \leq 0.05$. This figure has been reprinted from Monaghan *et al.*[2] with permission. Please click here to view a larger version of this figure.

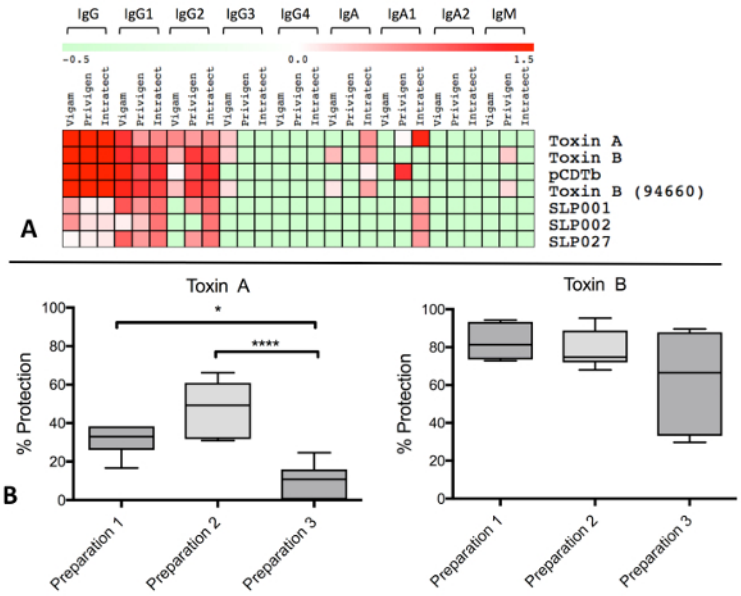

Figure 5: Immune reactivity and neutralizing effect of IVIg to *C.difficile* antigens. A. This image shows the reactivity of multi-isotype specific antibodies to *C. difficile* antigens in commercial intravenous immunoglobulin (IVIg) preparations. The heat map illustrates the levels of specific antibody isotypes (IgG, IgG1, IgG2, IgG3, IgG4, IgA, IgA1, IgA2, and IgM) in three commercially available preparations (see **Table of Materials**) against seven *C. difficile* antigens [toxin A (200 µg/mL), toxin B (100 µg/mL), pCDTb (200 µg/mL), toxin B (CCUG 20309; 90 µg/mL), and surface layer proteins (SLPs) 001, 002, and 027 (all 200 µg/mL)] using protein microarray technology. The color code of the heat map is as follows: green (low) to red (high) signal intensity. The signal values represented on the color scale for the heat map are log2-transformed from the arbitrary fluorescence units (AFU). Please note that the AFU has more recently been superseded by the descriptor standardized signals[2]. The total IgG, IgG1, and IgG2 isotypes gave the highest binding reactivities against toxin A, toxin B, binary toxin (pCDTb), and toxin B (CCUG 20309). **B**. These plots show the IVIg neutralization efficacy against the native *C. difficile* whole toxins A and B. They give the percentage of the protective neutralization effect of commercial IVIg products against *C. difficile* toxins A and B. Each plot represents the median of triplicate experiments at 1:100 dilution. IVIg preparation 1 exhibits the lowest protective effect compared to IVIg preparations 2 and 3, particularly against toxin A. **** $P \leq 0.0001$; * $P \leq 0.05$ (one-way analysis of variance). This figure has been modified from Negm *et al.*[3] with permission. Please click here to view a larger version of this figure.

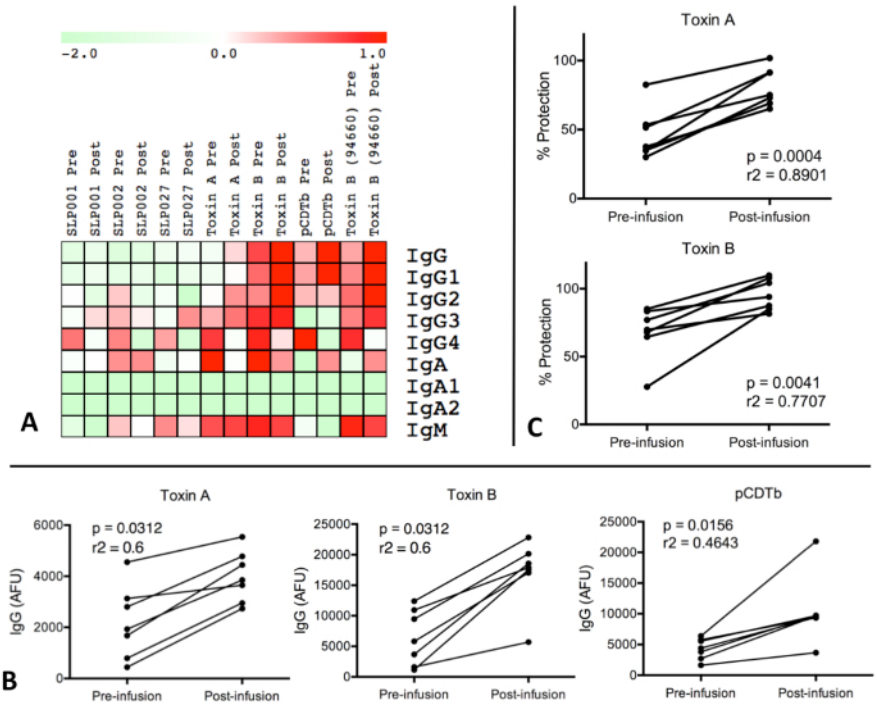

Figure 6: Immune reactivity and neutralizing effect of patient's sera to *C. difficile* antigens. A. This figure shows a comparison of antibody reactivities against *C. difficile* proteins in patients' sera before and after IVIg infusion. The heat map illustrates the expression level of the isotypes (IgG, IgG1, IgG2, IgG3, IgG4, IgA, IgA1, IgA2, and IgM) in serum samples in seven patients before and after IVIg infusion against seven *C. difficile* antigens [toxin A (200 µg/mL), toxin B (100 µg/mL), pCDTb (200 µg/mL), toxin B (CCUG 20309; 90 µg/mL), and SLPs 001, 002, and 027 (all 200 µg/mL)] using protein microarray technology. The color code of the heat map is as follows: green (low) to red (high) signal intensity. The signal values represented on the color scale for the heat map are log2-transformed from the AFU. Please note that the AFU has more recently been superseded by standardized signals[2]. There was a post-infusion enhancement of the total IgG, IgG1, IgG2, and IgG3 reactivities to toxin A, toxin B, and pCDTb. **B**. This image shows the IgG responses to toxin A, toxin B, and binary toxin (pCDTb), pre- and post-IVIg administration. The total IgG levels against all toxins show a significant increase following the IVIg administration (using the Wilcoxon signed-rank test). Each plot represents the median of triplicate experiments at 1:10 dilution. **C**. These plots show the neutralization effect against native *C. difficile* toxins A and B following IVIg administration. A comparison of pre- and post-infusion neutralizing antibody activities shows an enhanced protective effect after the IVIg infusions against the native *C. difficile* toxins A and B. Each plot represents the median of triplicate experiments at 1:10 dilution. A significant increase in the protective effect against toxins A and B was noted in the patient sera tested post-IVIg infusion (using the Wilcoxon signed-rank test). This figure has been reprinted from Negm *et al.*[3] with permission. Please click here to view a larger version of this figure.

Reproducibility	**Toxin A**	**Toxin B**	**SLP001**	**SLP002**	**SLP027**	**Toxin B CCUG 20309**	**pCDT**b
Intra-assay	7.70%	6.40%	7.40%	5.10%	7.60%	7%	3.70%
Inter-assay	9.10%	9.10%	7.40%	11.20%	12.8	9.70%	12.50%

Table 1: Microarray intra-assay and inter-assay precision[1]. Microarray intra- and inter-assay variabilities were calculated using the sera of 7 patients. Identical samples were assayed on each of two slides at two independent time points. All antigens (n = 7 test and n = 2 controls) were spotted in replicates of five on each array.

	Purified protein concentration	**Serum dilution**	**Secondary antibody dilution**
IgG	50 µg/ml	1/500	1/20000
IgG1	200 µg/ml	1/100	1/5000
IgG2	200 µg/ml	1/100	1/10000
IgG3	200 µg/ml	1/100	1/5000
IgG4	200 µg/ml	1/100	1/5000
IgA	50 µg/ml	1/100	1/5000
IgA1	200 µg/ml	1/100	1/10000
IgA2	200 µg/ml	1/100	1/5000
IgM	50 µg/ml	1/500	1/20000

Table 2: **List of immunoglobulins used in this study.** The Igs are shown with the relevant purified protein concentrations (µg/mL) and dilutions for serum and secondary antibodies.

Discussion

In this protocol, we have shown that microarray is a suitable platform for defining humoral immune responses to *C. difficile* protein antigens in patient sera (**Figures 3** and **6**) and commercial preparations of IVIg (**Figure 5**). We have also demonstrated that the microarray technique performs well when compared to conventional ELISA (**Figure 2**) and shows excellent reproducibility, with intra- and inter-assay variabilities falling within acceptable limits of precision (**Table 1**).

Critical steps:
A number of critical steps must be followed when building any successful antigen microarray platform. Initially, it is extremely important to run QC experiments. In the QC experiments, different parameters should be evaluated, such as the selection of the appropriate surface chemistry, printing buffers, blocking buffers, dilutions of the serum samples and the secondary antibodies. These experiments must be performed with the aim of delivering a well-validated and reliable technique[38,39].

The selection of a suitable protein immobilization process is one of the most important steps in microarray analyses, to ensure a high-quality performance of the tested aminosilane slides, as seen in this study. It is crucial to observe regular and circular spot morphology, high and specific signal intensity, and a clear background. Another factor affecting microarray performance is the printing buffer, which is equally important to achieve the desired surface chemistry to produce uniform and regular spots on the slide.

It is important to select the correct settings for printing as this will allow the optimal size and shape of a spot to be printed. For example, the humidity level should be maintained around 55% during printing, because without humidity the rate of evaporation in the microarray chamber is increased and fewer spots are printed.

Non-specific protein binding is an additional factor affecting the background and spot signals; therefore, appropriate selection of a blocking buffer could reduce any non-specific binding, leading to improved sensitivity and accuracy of the array data[39].

Modifications and troubleshooting:
Antigens and serum samples must be aliquoted and stored in smaller storage tubes in the freezer until use, which helps avoid repeated multiple freeze-thaw cycles, which can deteriorate the signal strength of the array. To enhance the chances of a successful experiment, all the reagents must be prepared fresh and be filtered. Cleaning the arrayer before printing and checking the settings are required. Moreover, the slide holder must be kept clean to minimize background noise.

Limitations:
The application of protein microarrays is currently hampered by the stringent demand on surface chemistry in protein microarray fabrication. Here the great variation in the chemical and physical properties of protein molecules necessitates custom-designing unique surface chemistry for different classes of protein and antibody molecules[40]. Other technical challenges facing the widespread implementation of such technology include the need for expensive specialized equipment and software with allocated bench-space, as well as consideration of maintenance charges, complex data analysis, relative protein quantification, and critically access to purified antigens.

Significance of method with respect to existing/alternative methods:
Microarray technology is similar in principle to ELISA, Meso Scale Discovery, or Luminex immunoassays, but is customizable, can be scaled up to achieve statistical power, relies on only microliter quantities of precious samples, and has the ability to screen sera for multiple protein reactivities. It is therefore particularly suitable for large-scale, historical serum banks and biomarker discovery and screening.

Future applications or directions of method:
Future efforts will be directed towards optimizing the assay for other samples (cell supernatants), using the assay as a prognostic tool for patient stratification, externally validating potential biomarkers using large cohorts in a double-blind fashion, and predicting and optimizing response to immunotherapy (mAb, vaccines). In the future, the microarray technology could be used in a hospital setting as a useful diagnostic tool or to monitor a drug treatment plan over a period of time.

Disclosures

None.

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