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Main title: Profiling Humoral Immune Responses to *Clostridium difficile*-
Specific Antigens by Protein Microarray Analysis

Running title: *C. difficile* Antigen-Specific Microarrays

Ola H Negm,1,2 Mohamed R Hamed,1,2 Elizabeth M Dilnot,1 Clifford C Shone,3 Izabela Marszalowska,4 Mark Lynch,4 Christine E Loscher,4 Laura J Edwards,5 Patrick J Tighe,1 Mark H Wilcox,6 and Tanya M Monaghan7

Address correspondence to Dr Tanya M Monaghan, tanya.monaghan@nottingham.ac.uk

1Immunology, School of Life Sciences, University of Nottingham, Nottingham, 2Medical Microbiology and Immunology, Faculty of Medicine, Mansoura University, Egypt, 3Public Health England, Salisbury, United Kingdom, 4Immunomodulation Research Group, Dublin City University, Dublin, Ireland, 5Faculty of Medicine and Health Sciences, University of Nottingham, Nottingham, United Kingdom, 6Leeds Institute for Molecular Medicine, University of Leeds, Leeds, United Kingdom, 7NIHR Nottingham Digestive Diseases Biomedical Research Unit, Nottingham, United Kingdom

Abstract

*Clostridium difficile* is an anaerobic, Gram positive and spore forming bacterium that is the leading worldwide infective cause of hospital-acquired and antibiotic-associated diarrhea. Several studies have reported associations between
humoral immunity and the clinical course of *C. difficile* infection (CDI). Host humoral immune responses are determined using conventional enzyme-linked immunosorbant assay (ELISA) techniques. Herein, we report the first use of a novel protein microarray assay to determine systemic IgG antibody responses against a panel of highly purified *C. difficile*-specific antigens, including native toxins A and B (TcdA and TcdB), recombinant fragments of toxins A and B (TxA4 and TxB4), ribotype-specific surface layer proteins (SLPs; 001, 002, 027) and control proteins (tetanus and candida). Microarrays were probed with sera from a total of 327 individuals with CDI, cystic fibrosis without diarrhea, and healthy controls. For all antigens, precision profiles demonstrated <10% coefficient of variation (CV). Significant correlation was observed between microarray and ELISA in the quantification of anti-toxin A and -B IgG. These results indicate that microarray is a suitable assay for defining humoral immune responses to *C. difficile* protein antigens and may have potential advantages in throughput, convenience and cost.

192 words

**Keywords.** *Clostridium difficile*, humoral immune responses, protein microarray

**Introduction**

*Clostridium difficile* is the leading worldwide infective cause of hospital-acquired and antibiotic-associated diarrhea, imposing a considerable financial burden on health service providers in both Europe and the USA [1-3]. Infection causes a spectrum of clinical presentations, ranging from an asymptomatic carrier state to severe fulminant colitis and death [4]. Following successful treatment, an
estimated 20-30% of patients with primary *C. difficile* infection (CDI) develop recurrence of symptoms, either caused by relapse of the original infection or reinfection with a new strain [5].

This anaerobic and spore-forming bacterium exerts its major pathological effects through two pro-inflammatory and cytotoxic protein exotoxins, TcdA (toxin A) and TcdB (toxin B) [6]. Non-toxin virulence factors such as surface layer proteins (SLPs) and cell wall proteins (CWPs) have also been described, and may play a role in disease expression [7-9].

The majority of healthy adults have detectable antibodies to *C. difficile* TcdA and TcdB in their serum that are thought to arise from colonization in infancy or from repeated exposure to *C. difficile* in adulthood from the environment [10-11]. Several clinical studies suggest that adaptive humoral immune responses, in particular to TcdA and TcdB, may influence clinical outcomes of CDI [12]. Most notably, a landmark study in 2000 reported that a low IgG titre to TcdA, but not TcdB, at the time of infection is associated with development of symptomatic disease [13]. More recently, the same group demonstrated an association between median IgG titres to TcdA and 30-day all-cause mortality [14]. Several reports have also assessed antibody responses following infection and shown protection against recurrence associated with antibody responses to TcdA, TcdB and several non-toxin antigens (Cwp66, Cwp84, FliC, FliD and the surface layer proteins) [15-18]. By contrast, other studies have reported that humoral immune
responses did not influence the clinical course of CDI [18-21]. These conflicting reports may be attributed to heterogeneity in study design and subject populations. Although the role of humoral immunity remains incompletely understood, vaccination strategies using inactivated toxins or recombinant toxin fragments are currently the subject of intense investigation [22-23]. More recently, the possibility of adding other vaccine targets such as surface-associated proteins and polysaccharides to toxin combinations is gaining traction, and could be of added value in the prevention of *C. difficile* colonization and disease transmission [22-23]. It is likely that the design of these next generation multicomponent vaccines targeting colonization, persistence and toxin production will stimulate the requirement for evaluating humoral immune responses to multiple antigens.

The enzyme-linked immunosorbant assay (ELISA) is the traditional method of accurately quantifying antibodies with different specificities in epidemiologic research and vaccine development as well as in the diagnosis of allergies, autoimmune and infectious diseases. We and other groups [13, 15, 18-20] have independently developed and used a traditional standardized ELISA format for the purposes of determining human specific IgG responses against *C. difficile* antigens (toxins). However, ELISA based tests can be time-consuming and require large quantities of both sample and reagents, thus limiting their potential for high-throughput use [23-24]. ELISA offers only monoplex data, or results of a single protein per assay (typically TcdA, TcdB or SLPs) and from a single *C.
difficult strain. Additional concerns include the lack of a uniform standard for
calibration purposes and thus no generally accepted way of expressing ELISA
units, as well as poor consistency between protocols and reagents, including
notably the quality and source of antigens. Moreover, the linear region of the
dynamic range is highly platform dependent.

Recently, protein microarrays, a miniaturized version of a sandwich ELISA, have
evolved as a promising tool for quantifying specific antibodies directed against
various microbial antigens in human sera, and may be an attractive alternative to
conventional ELISA assays in determining antigen-specific antibody responses
[25-31]. Microarray assays have potentially important advantages compared with
standard ELISA formats. These include a much increased capacity for
multiplexing detection of a range of specific antibodies due to the flexibility of
array printing of multiple antigens per array over a single protein, vastly reduced
requirements for antigens, serum and reagents, increased assay robustness due
to increased technical replication within each assay, multiple internal quality
control measures and improved quality control capabilities. The unique
capabilities of microarray including parallelism, high-throughput and
miniaturization are ideally suited to comprehensive investigation of the humoral
immune response to the entire proteome of an infectious agent consisting of
thousands of potential antigens, in a patient-specific manner [29]. Microarray
technology can also be applied to the development of improved serodiagnostic
tests, discovery of subunit vaccine antigen candidates, epidemiological research
and vaccine development, in addition to providing novel insights into infectious
disease and the immune system [29].

We have developed and validated a novel customized microarray platform that
enables the simultaneous quantification of systemic IgG immune responses to a
7-plex panel of highly purified \textit{C. difficile}-specific virulence factors, including
whole toxins A and B, recombinant fragments of toxin A (TxA4) and toxin B
(TxB4), type-specific surface layer proteins and suitable control proteins. We
compared the performance of the microarray technique with a conventional
ELISA using an established panel of sera.

\textbf{Materials and Methods}

\textbf{Microbial proteins and serum samples}

Highly purified whole toxins A and B (toxinotype 0, strain VPI10463, ribotype 087)
in addition to recombinant toxin fragments TxA4 and TxB4, (comprising central
and receptor binding domains and both based on toxinotype 0 sequences), were
obtained from Public Health England, UK (Dr Clifford Shone). Purified
polymerase chain reaction (PCR) ribotype-specific native whole SLPs (001, 002,
027) were provided by Dublin City University, Eire (Professor Christine Loscher).
Positive controls incorporated on each plate included tetanus toxoid and lysates
from \textit{Candida} albicans containing the cytoplasm and cell wall.
Negative controls included spotted printing buffer (PBS Trehalose Tween) and no serum (blank) on each array.

Banked sera from adult patients with CDI [n=150; median age 67 years (range 19-98 years), a group of patients with cystic fibrosis (CF) without diarrhea [n=17 where 2 of these patients were found to be asymptomatic carriers; median age 28 (19-49 years)] and healthy controls [n=67; median age 36 years (22-65 years)] were used to investigate the ability of the microarray assay to detect the presence or absence of IgG directed against *C. difficile* microbial and control antigens. Adult healthy donors were recruited from within the hospital and University workforce setting. All the patients in the CDI group had diarrhea (defined as a change in bowel habit with 3 or more unformed stools per day for at least 48 hours) and positive stool *C. difficile* toxin test. Asymptomatic carriers were defined as those without diarrhea, but had a positive stool culture for *C. difficile*. The diagnosis of CF had previously been made on the basis of a positive sweat test and/or demonstration of 2 known CF mutations and typical clinical features of disease (without a history of CDI). All subjects provided written informed consent under approvals granted by the Nottingham Research Ethics Committee.

**Preparation and processing of arrayed antigens**
Microbial antigens were diluted to 200μg/ml in printing buffer (PBS Trehalose
Tween) in a 384-well plate (Genetix) and spotted in quadruplicate in a 16 x 16
array format onto poly-L-Lysine-coated glass slides (Electron Microscopy
Sciences) using a Biorobotics MicroGridII arrayer (Microgrid 610, Digilab,
Malborough, MA, USA) in addition to 15 human serial IgG dilutions (range 50
μg/ml – 3.05 ng/ml) to create a calibration curve. The slides were blocked with
5% BSA diluted in PBS-Tween (PBST; PBS containing 0.05% Tween-20) wash
buffer for 1 hour at room temperature (RT) with shaking. After washing 5 times
for 3 minutes each with PBST, all slides were incubated with sera diluted 1:500 in
antibody diluent (Dako) for 1 hour. Following washing, the slides were incubated
with biotinylated anti-human IgG (Vector Labs) diluted 1:20,000 in antibody
diluent for 1 hour. After further washing, slides were incubated with Streptavidin
Cy5 (ebioscience) diluted 1:2000 in 5% BSA for 15 minutes. After a final wash
with PBST followed by distilled water, slides were dried by centrifugation at 500 g
for 4 minutes. Unless stated otherwise, all wash steps were carried out at RT with
shaking. Slides were scanned using a GenePix 4200AL scanner, a PMT of 450
and 100% power. The resultant TIFF images were processed with Axon Genepix
Pro-6 Microarray Image Analysis software (Molecular Services Inc.) to obtain
fluorescence data for each feature and generate gpr files. Protein signals were
finally determined with background subtraction using RPPanalyzer, a module
within the R statistical language on the CRAN ([http://cran.r-project.org/](http://cran.r-project.org/)) [33]

Statistical analysis
Antibody levels were calculated using GraphPad Prism 6.0 Software. As data collected for antibody measurements were not normally distributed, non-parametric tests were employed with medians and ranges calculated. For comparison of multiple groups, Kruskall Wallis one-way ANOVA was used with Dunn’s post-test. Correlation was evaluated using the Spearman rank correlation coefficient test. *P* values of <0.05 were considered to represent statistically significant differences.

Results

Quality control measures

Internal QC measures on each array were devised to support inter-assay normalization, assay performance and data acquisition machine performance monitoring. These measures included the addition of a replicated serial dilution of human IgG to verify function of the detection system and provide a standard curve of human IgG against which antibody responses could be calibrated. Antigens from 2 known human pathogens (tetanus toxoid and *Candida albicans*), where the majority of normal individuals would be expected to have some existing protective antibody response were incorporated onto each array. These positive control antigens were examined for each array as an indicator of sample integrity. Figure 1 shows a plot of the responses seen in 327 serum samples for each of the 2 control antigens. Strong responses are seen to tetanus toxoid and *Candida albicans*. Negative controls were also incorporated onto each array as a further internal QC measure.
Microarray Intra- and Inter-assay Precision

Microarray intra-and inter-assay variability was calculated using the sera of 7 patients. Identical samples were assayed on each of two slides at two independent time points. All antigens were spotted in replicates of five on each array. In the case of intra-assay variation, all 7 test and 2 control antigens fell within acceptable limits of precision [coefficient of variation (CV) <10%; toxin A 7.76%, toxin B 6.39%, SLP001 7.44%, SLP002 5.19%, SLP027 7.64%, TxA4 7.03%, TxB4 3.71%, tetanus 4.21%, candida 8.28%]. The inter-assay coefficient of variation for each antigen was calculated as 7.76%, 6.39%, 7.44%, 5.19%, 7.64%, 7.03%, 3.71%, 4.21% and 8.26%, respectively.

Correlation between Microarray and ELISA assay results

Due to the lack of validated and commercially available quantitative and standardized IgG ELISAs targeting toxins A and B or other *C. difficile*-associated antigens, we compared specific IgG anti-toxin A and anti-toxin B antibody measurements generated by microarray versus previously obtained in-house indirect ELISA readings using the same patient test sera [21]. Spearman correlation coefficient was used to assess the level of agreement between the two platforms and results are visually represented in Figure 2. When comparing the microarray performance with the in-house ELISA assays, a good correlation
coefficient was observed for toxin A ($r= 0.7051; p<0.0001$) with a moderately good correlation for toxin B ($r= 0.5809 p<0.0001$).

Sensitivity and specificity

Sensitivity and specificity of individual and panels of antibody response to *C. difficile*-specific antigens were calculated for both the CDI and CF groups using selected age-matched samples and the same panel of 7 antigens (Toxin A, Toxin B, SLP001, SLP002, SLP027, TxA4 and TxB4) based upon the same cut-off of 95th percentile of the control samples. CF samples have a sensitivity and specificity of 75% and 100% respectively while the figures for CDI are 25% and 100% respectively.

Serum antibody reactivity profiles using microarray

A total of 327 serum samples were tested by microarray for the presence of specific IgG antibody. The microarray assay was able to detect specific antibody responses to all *C. difficile* antigens including recombinant toxin fragments tested (example response to native antigens demonstrated in Figure 3). The signals from positive control proteins (tetanus and candida) were similar in healthy control individuals, patients with CF and CDI. Each array also included negative controls (buffer only and no serum or blank) which gave no signal. The reactivity of these spots was routinely subtracted from all signals obtained from specific antigens. The microarrays detected significantly higher levels of specific
antibodies in the CF group across all *C. difficile* antigens tested compared with healthy control and with CDI sera (Figure 4). In the CDI group, antibody responses to whole (Figure 4A and B) and recombinant toxins A and B (Figure 5) did not differ compared with the healthy control group, but infected patients did exhibit significantly lower anti-SLP IgG levels (all ribotypes) compared with controls and with patients with CF (Figure 4C). No statistically significant differences were observed in specific antibody levels to any of the antigens comparing single and relapsing CDI sera (data not presented).

**Discussion**

Current knowledge of the complete antigen repertoire recognized by patients during CDI is sparse, limiting a detailed interrogation of immunity, exposure and hindering preclinical vaccine development. The goal of this study was to develop, validate and implement a novel protein microarray readout assay that allows the accurate, precise, and reproducible quantification of specific antibody responses to a selected panel of *C. difficile*-specific microbial antigens using a pre-existing bank of test sera.

This study represents, to our knowledge, the first report of highly purified anti-SLP 001, 002 and 027 IgG responses in a large cohort of patient sera and extends the usefulness of immunoassay techniques through simultaneous
examination of multiple *C. difficile*-specific antigens including toxins in one immunoassay layout. We demonstrate that serum *C. difficile* antigen-specific IgG antibody responses can be detected using this technique, and that the magnitude and breadth of response to individual specific microbial antigens differs greatly between individuals and patient groups.

Whilst our assay achieved excellent specificity for the target panel of antigens analysed, lower detection sensitivity was observed, particularly for the CDI group. Importantly, antibody-based serological assays are hampered by the high likelihood or prior exposure to micro-organisms encountered in the environment. In this regard, all populations previously exposed to *C. difficile* bacterial antigens will produce seropositive responses. Nevertheless, it should be possible to enhance sensitivity for specific antibody detection through probing the microarrays with a larger bank of longitudinal (acute and convalescent) test sera and/or activated B lymphocyte supernatant samples which secrete antigen-specific antibodies from patients with symptomatic CDI [21] and healthy controls who do not carry *C. difficile* in their stool. In addition, detection sensitivity may be improved by pooling several antigenic targets specific for *C. difficile*.

In contrast to previous studies asserting that development of symptomatic CDI may be correlated with low IgG titres to toxin A but not to toxin B, our data do not demonstrate any significant differences in IgG anti-toxin A or IgG anti-toxin B levels in CDI patients compared to healthy controls. Similarly, other investigators
have either reported no disparities or higher serum anti-toxin A IgG levels in CDI patients compared to controls [11, 19, 34]. Although the carriage rate of *C. difficile* in the control subjects was not known, the lack of difference may have arisen due to the fact that most of the healthy control subjects in this study were recruited from a pool of hospital and University co-workers. As such, it is likely that natural asymptomatic exposure to toxigenic *C. difficile* was a more common occurrence and thus may be due to transient colonization. Furthermore, we acknowledge that patients in the CDI group were also older compared to individuals in the CF and control groups. However, other investigators have shown that serum antibody levels were not affected by age [13, 15]. It will be of interest to determine whether specific qualitative and quantitative differences in T and B cell responses to *C. difficile* and its antigens account for higher prevalence of CDI in older populations.

Notably, significantly higher anti-toxin and anti-SLP IgG antibody concentrations in patients with CF (with no previous history of CDI) are likely to be due to more frequent contact with the toxins and SLPs of *C. difficile* which occur with colonization (especially after admission to hospital) and following antibiotic-mediated disruption of the protective resident microflora. Indeed, two of the patients with CF were asymptomatic carriers of *C. difficile*. Furthermore, this particular small cohort of CF patients also had additional risk factors for *C. difficile* colonization/infection, including tube feeding (n=5), and the use of proton pump inhibitors (n =14).
Whilst firm conclusions cannot be drawn because of the small number of subjects studied, our present data suggest that the host’s ability to mount a robust antibody response to multiple *C. difficile*-specific protein antigens as seen in the CF group, may help confer protection from developing symptomatic CDI. Protection from symptomatic CDI may be a higher order phenomenon related to patterns of antibody response as opposed to being attributable to any single antigenic target. Notably, CDI is rarely seen in CF patients despite the presence of multiple risk factors for infection, including frequent exposures to antibiotics and hospitals. Several studies have also shown that patients with CF are often asymptomatic carriers of *C. difficile* [35-38] with one recent report indicating that most strains carried by CF patients were non-toxigenic (77% versus 17%) [38]. It is also possible that colonization with non-toxigenic *C. difficile* may protect against colonization with toxin-producing strains and/or that differences in colonic mucus or the microbiome may also contribute to protection in the CF population. Understanding the role of the gut microbiota in programming the immune phenotype in the context of CF may offer a series of interactive windows that could be aligned to prevent CDI. Further detailed studies that aim to dissect the complex dialogue between the host, immune system and intestinal microbiota are currently underway in a larger cohort of CF patients. Limitations of the present study are the small sample of strain-specific bacterial proteins employed, unequal sample sizes in the different groups studies, lack of
age matching, the absence of colonizing/immunizing strain information, the study of only one isotype, the lack of antibody neutralization data and the absence of ELISA and microarray correlation data for the recombinant toxin fragments and non-toxin antigens examined. Whether or not the immunogenicity of these latter selected antigens contributes to CDI protection remains to be fully determined.

We observed a lower correlation between microarray and ELISA in the toxin B assays ($r=0.58; P<0.0001$). This finding was particularly apparent at increasing IgG anti-toxin B concentrations and may mean that ELISA lacks accuracy at higher specific anti-toxin B IgG concentrations. Discordance between both methods could also be an effect of the low throughput of ELISA that requires the samples to be analyzed in small batches over a longer period of time; by contrast, the high-throughput array platforms permits analysis of large sample cohorts under similar experimental conditions in a much more rapid time frame, likely enhancing result reproducibility. Discrepancies between both technologies may also have arisen due to variations in the quality of sera and toxins over time, especially when new batches/different sources of toxin were tested. These results also suggest that mapping temporal changes in serological responses to *C. difficile* may be best undertaken using high-throughput methods such as protein microarray.

In summary, we verify that this initial design and implementation of a protein microarray platform is well suited to identify, quantify and compare multiple specific antigenic responses following challenge by *C. difficile*. Given that
antigenic variation occurs between different strains, host responses may well vary according to which are the prevalent strains. High-throughput assays will be important in measuring the heterogeneity of host immune responses. Modifications of this microarray approach could be employed to expand the antigen targets to include proteins derived from multiple strains of *C. difficile* in addition to investigating multiple isotype specificities. The microarray platform could also be adapted to study cytokine/chemokine repertoires in response to infection or vaccination for large collections of individual patient sera. Optimised immunological marker panels are yet to be developed for predicting host responses to *C. difficile*. Before advancements can be made, more detailed careful studies in larger well defined prospective cohorts will be required before this *C. difficile* antigen-specific microarray assay can be used as a prognostic tool as well as tailoring interventional strategies. Nevertheless, protein microarrays have the potential to provide a more comprehensive antigen-specific humoral immune response profile in vaccinated or infected humans, that could find beneficial applications in large-scale sero-epidemiological, longitudinal and sero-surveillance analyses.

References


Figure Legends.

Figure 1. Testing sample integrity of all serum samples using two positive control antigens: Tetanus toxoid and Candida albicans antigen. Strong responses are seen to both antigens from all the samples.

Figure 2. Correlation between microarray and ELISA IgG anti-toxin A (A) and IgG anti-toxin B (B) antibody levels in patients with C. difficile infection and in patients with cystic fibrosis without a history of diarrhoea. Each dot represents a serum sample from an individual patient. Spearman correlation coefficient tests revealed significant agreement between both assay results (*** denotes P < 0.0001). Abbreviations: ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G.

Figure 3. Selected serum IgG responses to C. difficile proteins on microarray. Green (low) to red (high) signal intensity heat map representing the relative IgG
response to *C. difficile* immunoreactive antigens (native toxins A and B, and ribotype-specific surface layer proteins 001, 002, 027) in different serum samples from patients infected with *C. difficile*, CF patients and healthy controls.

Figure 4. Anti-toxin A (A) and anti-toxin B (B) IgG responses in healthy controls subjects and patients with *C. difficile* infection and a group of patients with cystic fibrosis and no history of diarrhoea. Differences between groups were calculated using the Kruskall-Wallis test followed by Dunn's post test for multiple comparisons. Horizontal lines in each graph represent the median. (C) Patients with cystic fibrosis had significantly higher levels of specific IgG antibody levels to toxins A and B compared to healthy controls and patients with *C. difficile* infection. In the microarray assays, there were significantly lower anti-SLP IgG levels across all ribotypes tested (001, 002, 027) in patients with *C. difficile* infection compared to patients with cystic fibrosis and healthy controls (*** denotes \( p \leq 0.001 \), ** denotes \( p \leq 0.01 \), * denotes \( p \leq 0.05 \)). Abbreviations: SLP, surface layer protein.

Figure 5. Anti-TxA4 and anti-TxB4 IgG responses in healthy controls, patients with *C. difficile* infection and in a CF group of patients with no history of diarrhoea. Differences between groups were calculated using the Kruskall-Wallis test followed by Dunn's post test for multiple comparisons. Horizontal lines in each graph represent the median. In the microarray assays, CF patients displayed significantly higher IgG levels against both recombinant toxin fragments compared to healthy controls and patients with *C. difficile* infection (*** denotes \( p \leq 0.001 \)).
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Correspondence: Dr Tanya M Monaghan; NIHR Nottingham Digestive Diseases, Biomedical Research Unit, Nottingham University Hospitals NHS Trust, Queen’s Medical Centre, E floor, West Block, Nottingham NG7 2UH. Tel: +44 (0)115 9249924, extension 70589; fax: +44 (0)115 9709955. Email: tanya.monaghan@nottingham.ac.uk