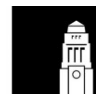

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1 **Main title: Profiling Humoral Immune Responses to *Clostridium difficile*-**
2 **Specific Antigens by Protein Microarray Analysis**
3
4 **Running title: *C. difficile* Antigen-Specific Microarrays**
5
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20
21 **Abstract**
22 *Clostridium difficile* is an anaerobic, Gram positive and spore forming bacterium
23 that is the leading worldwide infective cause of hospital-acquired and antibiotic-
24 associated diarrhea. Several studies have reported associations between

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25 humoral immunity and the clinical course of *C. difficile* infection (CDI). Host
26 humoral immune responses are determined using conventional enzyme-linked
27 immunosorbant assay (ELISA) techniques. Herein, we report the first use of a
28 novel protein microarray assay to determine systemic IgG antibody responses
29 against a panel of highly purified *C. difficile*-specific antigens, including native
30 toxins A and B (TcdA and TcdB), recombinant fragments of toxins A and B (TxA4
31 and TxB4), ribotype-specific surface layer proteins (SLPs; 001, 002, 027) and
32 control proteins (tetanus and candida). Microarrays were probed with sera from a
33 total of 327 individuals with CDI, cystic fibrosis without diarrhea, and healthy
34 controls. For all antigens, precision profiles demonstrated <10% coefficient of
35 variation (CV). Significant correlation was observed between microarray and
36 ELISA in the quantification of anti-toxin A and -B IgG. These results indicate that
37 microarray is a suitable assay for defining humoral immune responses to *C.*
38 *difficile* protein antigens and may have potential advantages in throughput,
39 convenience and cost.
40 **192 words**

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

42 **Introduction**

43 *Clostridium difficile* is the leading worldwide infective cause of hospital-acquired
44 and antibiotic-associated diarrhea, imposing a considerable financial burden on
45 health service providers in both Europe and the USA [1-3]. Infection causes a
46 spectrum of clinical presentations, ranging from an asymptomatic carrier state to
47 severe fulminant colitis and death [4]. Following successful treatment, an

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48 estimated 20-30% of patients with primary *C. difficile* infection (CDI) develop
49 recurrence of symptoms, either caused by relapse of the original infection or
50 reinfection with a new strain [5].
51
52 This anaerobic and spore-forming bacterium exerts its major pathological effects
53 through two pro-inflammatory and cytotoxic protein exotoxins, TcdA (toxin A) and
54 TcdB (toxin B) [6]. Non-toxin virulence factors such as surface layer proteins
55 (SLPs) and cell wall proteins (CWPs) have also been described, and may play a
56 role in disease expression [7-9].
57
58 The majority of healthy adults have detectable antibodies to *C. difficile* TcdA and
59 TcdB in their serum that are thought to arise from colonization in infancy or from
60 repeated exposure to *C. difficile* in adulthood from the environment [10-11].
61 Several clinical studies suggest that adaptive humoral immune responses, in
62 particular to TcdA and TcdB, may influence clinical outcomes of CDI [12]. Most
63 notably, a landmark study in 2000 reported that a low IgG titre to TcdA, but not
64 TcdB, at the time of infection is associated with development of symptomatic
65 disease [13]. More recently, the same group demonstrated an association
66 between median IgG titres to TcdA and 30-day all-cause mortality [14]. Several
67 reports have also assessed antibody responses following infection and shown
68 protection against recurrence associated with antibody responses to TcdA, TcdB
69 and several non-toxin antigens (Cwp66, Cwp84, FliC, FliD and the surface layer
70 proteins) [15-18]. By contrast, other studies have reported that humoral immune

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71 responses did not influence the clinical course of CDI [18-21]. These conflicting
72 reports may be attributed to heterogeneity in study design and subject
73 populations. Although the role of humoral immunity remains incompletely
74 understood, vaccination strategies using inactivated toxins or recombinant toxin
75 fragments are currently the subject of intense investigation [22-23]. More
76 recently, the possibility of adding other vaccine targets such as surface-
77 associated proteins and polysaccharides to toxin combinations is gaining traction,
78 and could be of added value in the prevention of *C. difficile* colonization and
79 disease transmission [22-23]. It is likely that the design of these next generation
80 multicomponent vaccines targeting colonization, persistence and toxin production
81 will stimulate the requirement for evaluating humoral immune responses to
82 multiple antigens.
83
84 The enzyme-linked immunosorbant assay (ELISA) is the traditional method of
85 accurately quantifying antibodies with different specificities in epidemiologic
86 research and vaccine development as well as in the diagnosis of allergies,
87 autoimmune and infectious diseases. We and other groups [13, 15, 18-20] have
88 independently developed and used a traditional standardized ELISA format for
89 the purposes of determining human specific IgG responses against *C. difficile*
90 antigens (toxins). However, ELISA based tests can be time-consuming and
91 require large quantities of both sample and reagents, thus limiting their potential
92 for high-throughput use [23-24]. ELISA offers only monoplex data, or results of a
93 single protein per assay (typically TcdA, TcdB or SLPs) and from a single *C.*

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94 *difficile* strain. Additional concerns include the lack of a uniform standard for
95 calibration purposes and thus no generally accepted way of expressing ELISA
96 units, as well as poor consistency between protocols and reagents, including
97 notably the quality and source of antigens. Moreover, the linear region of the
98 dynamic range is highly platform dependent.
99 Recently, protein microarrays, a miniaturized version of a sandwich ELISA, have
100 evolved as a promising tool for quantifying specific antibodies directed against
101 various microbial antigens in human sera, and may be an attractive alternative to
102 conventional ELISA assays in determining antigen-specific antibody responses
103 [25-31]. Microarray assays have potentially important advantages compared with
104 standard ELISA formats. These include a much increased capacity for
105 multiplexing detection of a range of specific antibodies due to the flexibility of
106 array printing of multiple antigens per array over a single protein, vastly reduced
107 requirements for antigens, serum and reagents, increased assay robustness due
108 to increased technical replication within each assay, multiple internal quality
109 control measures and improved quality control capabilities. The unique
110 capabilities of microarray including parallelism, high-throughput and
111 miniaturization are ideally suited to comprehensive investigation of the humoral
112 immune response to the entire proteome of an infectious agent consisting of
113 thousands of potential antigens, in a patient-specific manner [29]. Microarray
114 technology can also be applied to the development of improved serodiagnostic
115 tests, discovery of subunit vaccine antigen candidates, epidemiological research

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116 and vaccine development, in addition to providing novel insights into infectious
117 disease and the immune system [29].
118
119 We have developed and validated a novel customized microarray platform that
120 enables the simultaneous quantification of systemic IgG immune responses to a
121 7-plex panel of highly purified *C. difficile*-specific virulence factors, including
122 whole toxins A and B, recombinant fragments of toxin A (TxA4) and toxin B
123 (TxB4), type-specific surface layer proteins and suitable control proteins. We
124 compared the performance of the microarray technique with a conventional
125 ELISA using an established panel of sera.
126
127
128 **Materials and Methods**
129
130 **Microbial proteins and serum samples**
131 Highly purified whole toxins A and B (toxinotype 0, strain VPI10463, ribotype 087)
132 in addition to recombinant toxin fragments TxA4 and TxB4, (comprising central
133 and receptor binding domains and both based on toxinotype 0 sequences), were
134 obtained from Public Health England, UK (Dr Clifford Shone). Purified
135 polymerase chain reaction (PCR) ribotype-specific native whole SLPs (001, 002,
136 027) were provided by Dublin City University, Eire (Professor Christine Loscher).
137 Positive controls incorporated on each plate included tetanus toxoid and lysates
138 from *Candida* albicans containing the cytoplasm and cell wall.

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139 Negative controls included spotted printing buffer (PBS Trehalose Tween) and no
140 serum (blank) on each array.
141
142 Banked sera from adult patients with CDI [n=150; median age 67 years (range
143 19-98 years], a group of patients with cystic fibrosis (CF) without diarrhea [n=17
144 where 2 of these patients were found to be asymptomatic carriers; median age
145 28 (19-49 years)] and healthy controls [n=67; median age 36 years (22-65
146 years)] were used to investigate the ability of the microarray assay to detect the
147 presence or absence of IgG directed against *C. difficile* microbial and control
148 antigens. Adult healthy donors were recruited from within the hospital and
149 University workforce setting. All the patients in the CDI group had diarrhea
150 (defined as a change in bowel habit with 3 or more unformed stools per day for at
151 least 48 hours) and positive stool *C. difficile* toxin test. Asymptomatic carriers
152 were defined as those without diarrhea, but had a positive stool culture for *C.*
153 *difficile*. The diagnosis of CF had previously been made on the basis of a positive
154 sweat test and/or demonstration of 2 known CF mutations and typical clinical
155 features of disease (without a history of CDI). All subjects provided written
156 informed consent under approvals granted by the Nottingham Research Ethics
157 Committee.
158
159 **Preparation and processing of arrayed antigens**
160

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161 Microbial antigens were diluted to 200µg/ml in printing buffer (PBS Trehalose
162 Tween) in a 384-well plate (Genetix) and spotted in quadruplicate in a 16 x 16
163 array format onto poly-L-Lysine-coated glass slides (Electron Microscopy
164 Sciences) using a Biorobotics MicroGridII arrayer (Microgrid 610, Digilab,
165 Malborough, MA, USA) in addition to 15 human serial IgG dilutions (range 50
166 µg/ml – 3.05 ng/ml) to create a calibration curve. The slides were blocked with
167 5% BSA diluted in PBS-Tween (PBST; PBS containing 0.05% Tween-20) wash
168 buffer for 1 hour at room temperature (RT) with shaking. After washing 5 times
169 for 3 minutes each with PBST, all slides were incubated with sera diluted 1:500 in
170 antibody diluent (Dako) for 1 hour. Following washing, the slides were incubated
171 with biotinylated anti-human IgG (Vector Labs) diluted 1:20,000 in antibody
172 diluent for 1 hour. After further washing, slides were incubated with Streptavidin
173 Cy5 (ebioscience) diluted 1:2000 in 5% BSA for 15 minutes. After a final wash
174 with PBST followed by distilled water, slides were dried by centrifugation at 500 *g*
175 for 4 minutes. Unless stated otherwise, all wash steps were carried out at RT with
176 shaking. Slides were scanned using a GenePix 4200AL scanner, a PMT of 450
177 and 100% power. The resultant TIFF images were processed with Axon Genepix
178 Pro-6 Microarray Image Analysis software (Molecular Services Inc.) to obtain
179 fluorescence data for each feature and generate gpr files. Protein signals were
180 finally determined with background subtraction using RPPanalyzer, a module
181 within the R statistical language on the CRAN (http://cran.r-project.org/) [33]
182

183 **Statistical analysis**

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184 Antibody levels were calculated using GraphPad Prism 6.0 Software. As data
185 collected for antibody measurements were not normally distributed, non-
186 parametric tests were employed with medians and ranges calculated. For
187 comparison of multiple groups, Kruskall Wallis one-way ANOVA was used with
188 Dunn's post-test. Correlation was evaluated using the Spearman rank correlation
189 coefficient test. P values of <0.05 were considered to represent statistically
190 significant differences.
191
192 **Results**
193 *Quality control measures*
194 Internal QC measures on each array were devised to support inter-assay
195 normalization, assay performance and data acquisition machine performance
196 monitoring. These measures included the addition of a replicated serial dilution of
197 human IgG to verify function of the detection system and provide a standard
198 curve of human IgG against which antibody responses could be calibrated.
199 Antigens from 2 known human pathogens (tetanus toxoid and *Candida albicans*),
200 where the majority of normal individuals would be expected to have some
201 existing protective antibody response were incorporated onto each array. These
202 positive control antigens were examined for each array as an indicator of sample
203 integrity. Figure 1 shows a plot of the responses seen in 327 serum samples for
204 each of the 2 control antigens. Strong responses are seen to tetanus toxoid and
205 *Candida albicans*. Negative controls were also incorporated onto each array as a
206 further internal QC measure.

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207

208 *Microarray Intra- and Inter-assay Precision*

209

210 Microarray intra-and inter-assay variability was calculated using the sera of 7
211 patients. Identical samples were assayed on each of two slides at two
212 independent time points. All antigens were spotted in replicates of five on each
213 array. In the case of intra-assay variation, all 7 test and 2 control antigens fell
214 within acceptable limits of precision [coefficient of variation (CV) <10%; toxin A
215 7.76%, toxin B 6.39%, SLP001 7.44%, SLP002 5.19%, SLP027 7.64%, TxA4
216 7.03%, TxB4 3.71%, tetanus 4.21%, candida 8.28%]. The inter-assay coefficient
217 of variation for each antigen was calculated as 7.76%, 6.39%, 7.44%, 5.19%,
218 7.64%, 7.03%, 3.71%, 4.21% and 8.26%, respectively.

219

220 *Correlation between Microarray and ELISA assay results*

221

222 Due to the lack of validated and commercially available quantitative and
223 standardized IgG ELISAs targeting toxins A and B or other *C. difficile*-associated
224 antigens, we compared specific IgG anti-toxin A and anti-toxin B antibody
225 measurements generated by microarray versus previously obtained in-house
226 indirect ELISA readings using the same patient test sera [21]. Spearman
227 correlation coefficient was used to assess the level of agreement between the
228 two platforms and results are visually represented in Figure 2. When comparing
229 the microarray performance with the in-house ELISA assays, a good correlation

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230 coefficient was observed for toxin A (r= 0.7051; p<0.0001) with a moderately
231 good correlation for toxin B (r= 0.5809 p<0.0001).
232
233 *Sensitivity and specificity*
234 Sensitivity and specificity of individual and panels of antibody response to *C.*
235 *difficile*-specific antigens were calculated for both the CDI and CF groups using
236 selected age-matched samples and the same panel of 7 antigens (Toxin A, Toxin
237 B, SLP001, SLP002, SLP027, TxA4 and TxB4) based upon the same cut-off of
238 95th percentile of the control samples. CF samples have a sensitivity and
239 specificity of 75% and 100% respectively while the figures for CDI are 25% and
240 100% respectively.
241
242 *Serum antibody reactivity profiles using microarray*
243
244 A total of 327 serum samples were tested by microarray for the presence of
245 specific IgG antibody. The microarray assay was able to detect specific antibody
246 responses to all *C. difficile* antigens including recombinant toxin fragments tested
247 (example response to native antigens demonstrated in Figure 3). The signals
248 from positive control proteins (tetanus and candida) were similar in healthy
249 control individuals, patients with CF and CDI. Each array also included negative
250 controls (buffer only and no serum or blank) which gave no signal. The reactivity
251 of these spots was routinely subtracted from all signals obtained from specific
252 antigens. The microarrays detected significantly higher levels of specific

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253 antibodies in the CF group across all *C. difficile* antigens tested compared with
254 healthy control and with CDI sera (Figure 4). In the CDI group, antibody
255 responses to whole (Figure 4A and B) and recombinant toxins A and B (Figure 5)
256 did not differ compared with the healthy control group, but infected patients did
257 exhibit significantly lower anti-SLP IgG levels (all ribotypes) compared with
258 controls and with patients with CF (Figure 4C). No statistically significant
259 differences were observed in specific antibody levels to any of the antigens
260 comparing single and relapsing CDI sera (data not presented).
261
262
263 **Discussion**
264
265 Current knowledge of the complete antigen repertoire recognized by patients
266 during CDI is sparse, limiting a detailed interrogation of immunity, exposure and
267 hindering preclinical vaccine development. The goal of this study was to develop,
268 validate and implement a novel protein microarray readout assay that allows the
269 accurate, precise, and reproducible quantification of specific antibody responses
270 to a selected panel of *C. difficile*-specific microbial antigens using a pre-existing
271 bank of test sera.
272
273 This study represents, to our knowledge, the first report of highly purified anti-
274 SLP 001, 002 and 027 IgG responses in a large cohort of patient sera and
275 extends the usefulness of immunoassay techniques through simultaneous

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276 examination of multiple *C. difficile*-specific antigens including toxins in one
277 immunoassay layout. We demonstrate that serum *C. difficile* antigen-specific IgG
278 antibody responses can be detected using this technique, and that the magnitude
279 and breadth of response to individual specific microbial antigens differs greatly
280 between individuals and patient groups.
281
282 Whilst our assay achieved excellent specificity for the target panel of antigens
283 analysed, lower detection sensitivity was observed, particularly for the CDI group.
284 Importantly, antibody-based serological assays are hampered by the high
285 likelihood or prior exposure to micro-organisms encountered in the environment.
286 In this regard, all populations previously exposed to *C. difficile* bacterial antigens
287 will produce seropositive responses. Nevertheless, it should be possible to
288 enhance sensitivity for specific antibody detection through probing the
289 microarrays with a larger bank of longitudinal (acute and convalescent) test sera
290 and/or activated B lymphocyte supernatant samples which secrete antigen-
291 specific antibodies from patients with symptomatic CDI [21] and healthy controls
292 who do not carry *C. difficile* in their stool. In addition, detection sensitivity may be
293 improved by pooling several antigenic targets specific for *C. difficile.*
294
295 In contrast to previous studies asserting that development of symptomatic CDI
296 may be correlated with low IgG titres to toxin A but not to toxin B, our data do not
297 demonstrate any significant differences in IgG anti-toxin A or IgG anti-toxin B
298 levels in CDI patients compared to healthy controls. Similarly, other investigators

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299 have either reported no disparities or higher serum anti-toxin A IgG levels in CDI
300 patients compared to controls [11, 19, 34]. Although the carriage rate of *C.*
301 *difficile* in the control subjects was not known, the lack of difference may have
302 arisen due to the fact that most of the healthy control subjects in this study were
303 recruited from a pool of hospital and University co-workers. As such, it is likely
304 that natural asymptomatic exposure to toxigenic *C. difficile* was a more common
305 occurrence and thus may be due to transient colonization. Furthermore, we
306 acknowledge that patients in the CDI group were also older compared to
307 individuals in the CF and control groups. However, other investigators have
308 shown that serum antibody levels were not affected by age [13, 15]. It will be of
309 interest to determine whether specific qualitative and quantitative differences in T
310 and B cell responses to *C. difficile* and its antigens account for higher prevalence
311 of CDI in older populations.
312
313 Notably, significantly higher anti-toxin and anti-SLP IgG antibody concentrations
314 in patients with CF (with no previous history of CDI) are likely to be due to more
315 frequent contact with the toxins and SLPs of *C. difficile* which occur with
316 colonization (especially after admission to hospital) and following antibiotic-
317 mediated disruption of the protective resident microflora. Indeed, two of the
318 patients with CF were asymptomatic carriers of *C. difficile*. Furthermore, this
319 particular small cohort of CF patients also had additional risk factors for *C.*
320 *difficile* colonization/infection, including tube feeding (n=5), and the use of proton
321 pump inhibitors (n =14).

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322

323 Whilst firm conclusions cannot be drawn because of the small number of subjects
324 studied, our present data suggest that the host's ability to mount a robust
325 antibody response to multiple *C. difficile*-specific protein antigens as seen in the
326 CF group, may help confer protection from developing symptomatic CDI.
327 Protection from symptomatic CDI may be a higher order phenomenon related to
328 patterns of antibody response as opposed to being attributable to any single
329 antigenic target. Notably, CDI is rarely seen in CF patients despite the presence
330 of multiple risk factors for infection, including frequent exposures to antibiotics
331 and hospitals. Several studies have also shown that patients with CF are often
332 asymptomatic carriers of *C. difficile* [35-38] with one recent report indicating that
333 most strains carried by CF patients were non-toxigenic (77% versus 17%) [38]. It
334 is also possible that colonization with non-toxigenic *C. difficile* may protect
335 against colonization with toxin-producing strains and/or that differences in colonic
336 mucus or the microbiome may also contribute to protection in the CF population.
337 Understanding the role of the gut microbiota in programming the immune
338 phenotype in the context of CF may offer a series of interactive windows that
339 could be aligned to prevent CDI. Further detailed studies that aim to dissect the
340 complex dialogue between the host, immune system and intestinal microbiota are
341 currently underway in a larger cohort of CF patients.

342

343 Limitations of the present study are the small sample of strain-specific bacterial
344 proteins employed, unequal sample sizes in the different groups studies, lack of

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345 age matching, the absence of colonizing/immunizing strain information, the study
346 of only one isotype, the lack of antibody neutralization data and the absence of
347 ELISA and microarray correlation data for the recombinant toxin fragments and
348 non-toxin antigens examined. Whether or not the immunogenicity of these latter
349 selected antigens contributes to CDI protection remains to be fully determined.
350 We observed a lower correlation between microarray and ELISA in the toxin B
351 assays (r=0.58; P<0.0001). This finding was particularly apparent at increasing
352 IgG anti-toxin B concentrations and may mean that ELISA lacks accuracy at
353 higher specific anti-toxin B IgG concentrations. Discordance between both
354 methods could also be an effect of the low throughput of ELISA that requires the
355 samples to be analyzed in small batches over a longer period of time; by
356 contrast, the high-throughput array platforms permits analysis of large sample
357 cohorts under similar experimental conditions in a much more rapid time frame,
358 likely enhancing result reproducibility. Discrepancies between both technologies
359 may also have arisen due to variations in the quality of sera and toxins over time,
360 especially when new batches/different sources of toxin were tested. These
361 results also suggest that mapping temporal changes in serological responses to
362 *C. difficile* may be best undertaken using high-throughput methods such as
363 protein microarray.
364
365 In summary, we verify that this initial design and implementation of a protein
366 microarray platform is well suited to identify, quantify and compare multiple
367 specific antigenic responses following challenge by *C. difficile*. Given that

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368 antigenic variation occurs between different strains, host responses may well
369 vary according to which are the prevalent strains. High-throughput assays will be
370 important in measuring the heterogeneity of host immune responses.
371 Modifications of this microarray approach could be employed to expand the
372 antigen targets to include proteins derived from multiple strains of *C. difficile* in
373 addition to investigating multiple isotype specificities. The microarray platform
374 could also be adapted to study cytokine/chemokine repertoires in response to
375 infection or vaccination for large collections of individual patient sera. Optimised
376 immunological marker panels are yet to be developed for predicting host
377 responses to *C. difficile*. Before advancements can be made, more detailed
378 careful studies in larger well defined prospective cohorts will be required before
379 this *C. difficile* antigen-specific microarray assay can be used as a prognostic tool
380 as well as tailoring interventional strategies. Nevertheless, protein microarrays
381 have the potential to provide a more comprehensive antigen-specific humoral
382 immune response profile in vaccinated or infected humans, that could find
383 beneficial applications in large-scale sero-epidemiological, longitudinal and sero-
384 surveillance analyses
385

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513 **Figure Legends.**
514 **Figure 1.** Testing sample integrity of all serum samples using two positive control
515 antigens: Tetanus toxoid and *Candida albicans* antigen. Strong responses are
516 seen to both antigens from all the samples.
517 **Figure 2.** Correlation between microarray and ELISA IgG anti-toxin A (A) and
518 IgG anti-toxin B (B) antibody levels in patients with *C. difficile* infection and in
519 patients with cystic fibrosis without a history of diarrhoea. Each dot represents a
520 serum sample from an individual patient. Spearman correlation coefficient tests
521 revealed significant agreement between both assay results (*** denotes $P <$
522 0.0001). Abbreviations: ELISA, enzyme-linked immunosorbent assay; IgG,
523 immunoglobulin G.
524 **Figure 3.** Selected serum IgG responses to *C. difficile* proteins on microarray.
525 Green (low) to red (high) signal intensity heat map representing the relative IgG

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526 response to *C. difficile* immunoreactive antigens (native toxins A and B, and
527 ribotype-specific surface layer proteins 001, 002, 027) in different serum samples
528 from patients infected with *C. difficile*, CF patients and healthy controls.

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

541 **Figure 5.** Anti-TxA4 and anti-TxB4 IgG responses in healthy controls, patients
542 with *C. difficile* infection and in a CF group of patients with no history of
543 diarrhoea. Differences between groups were calculated using the Kruskall-Wallis
544 test followed by Dunn's post test for multiple comparisons. Horizontal lines in
545 each graph represent the median. In the microarray assays, CF patients
546 displayed significantly higher IgG levels against both recombinant toxin
547 fragments compared to healthy controls and patients with *C. difficile* infection (***
548 denotes $p \leq 0.001$) .

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Footnote
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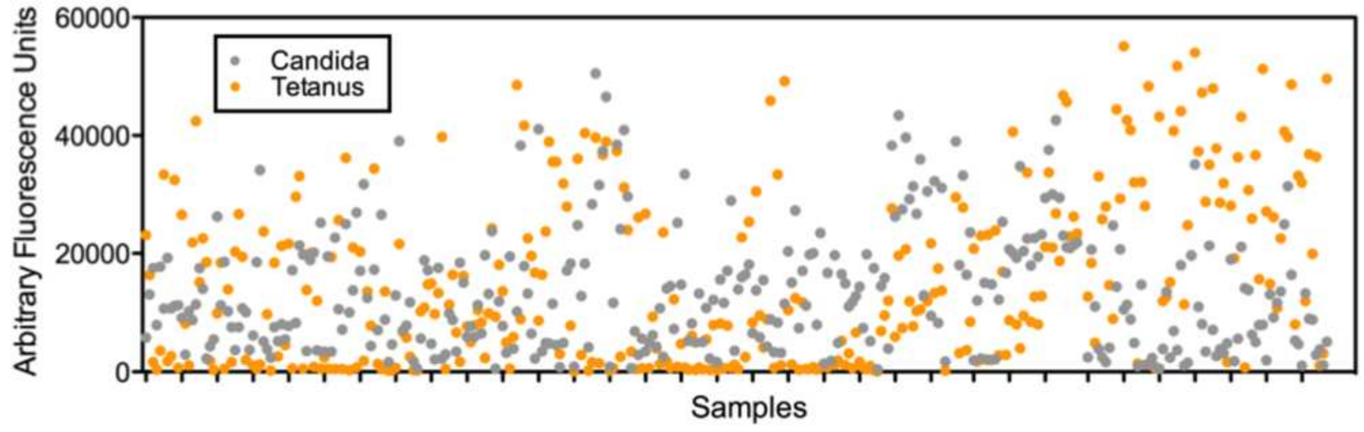

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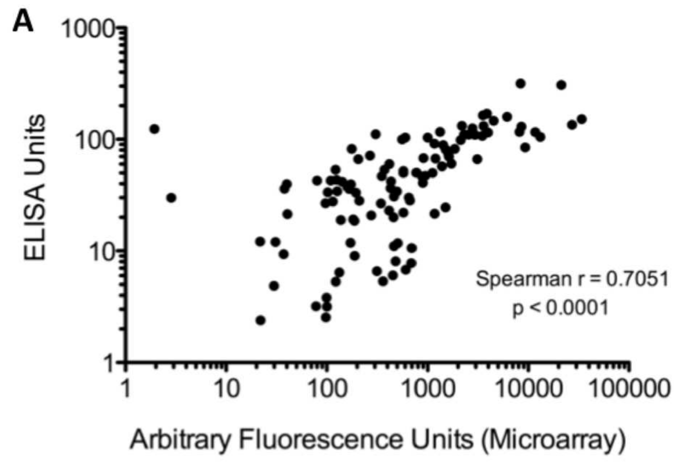

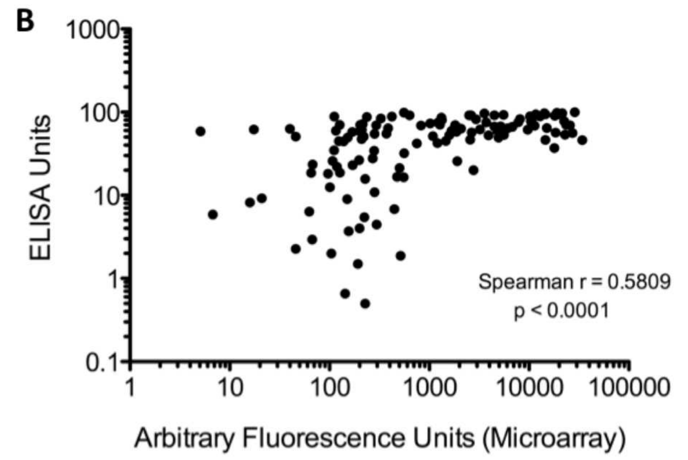

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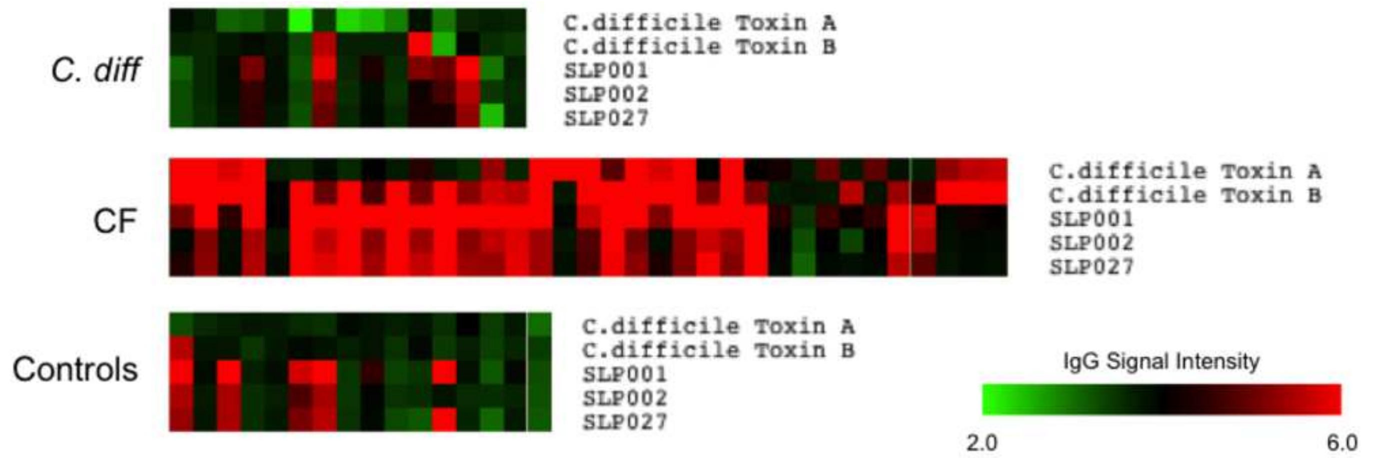

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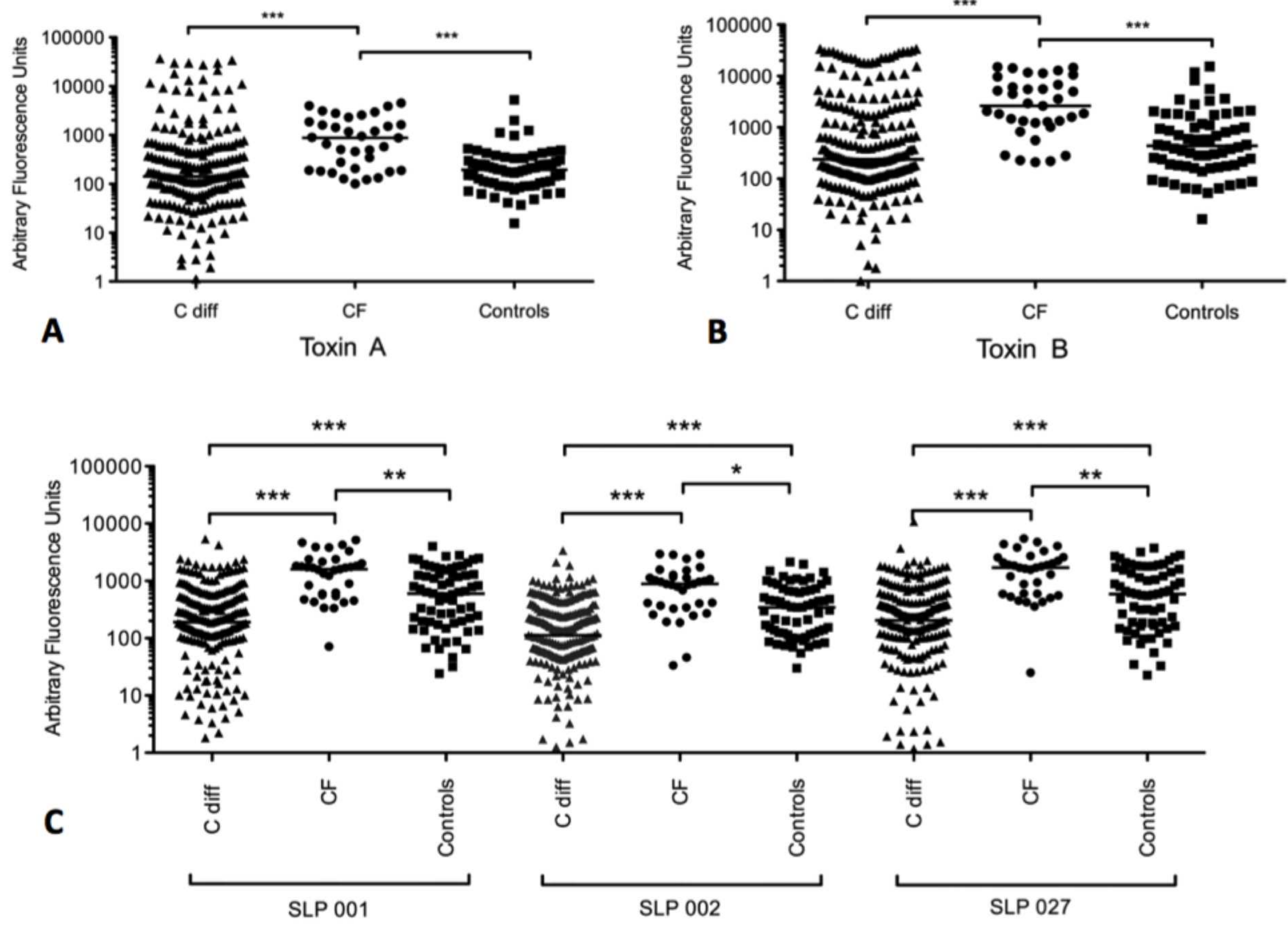

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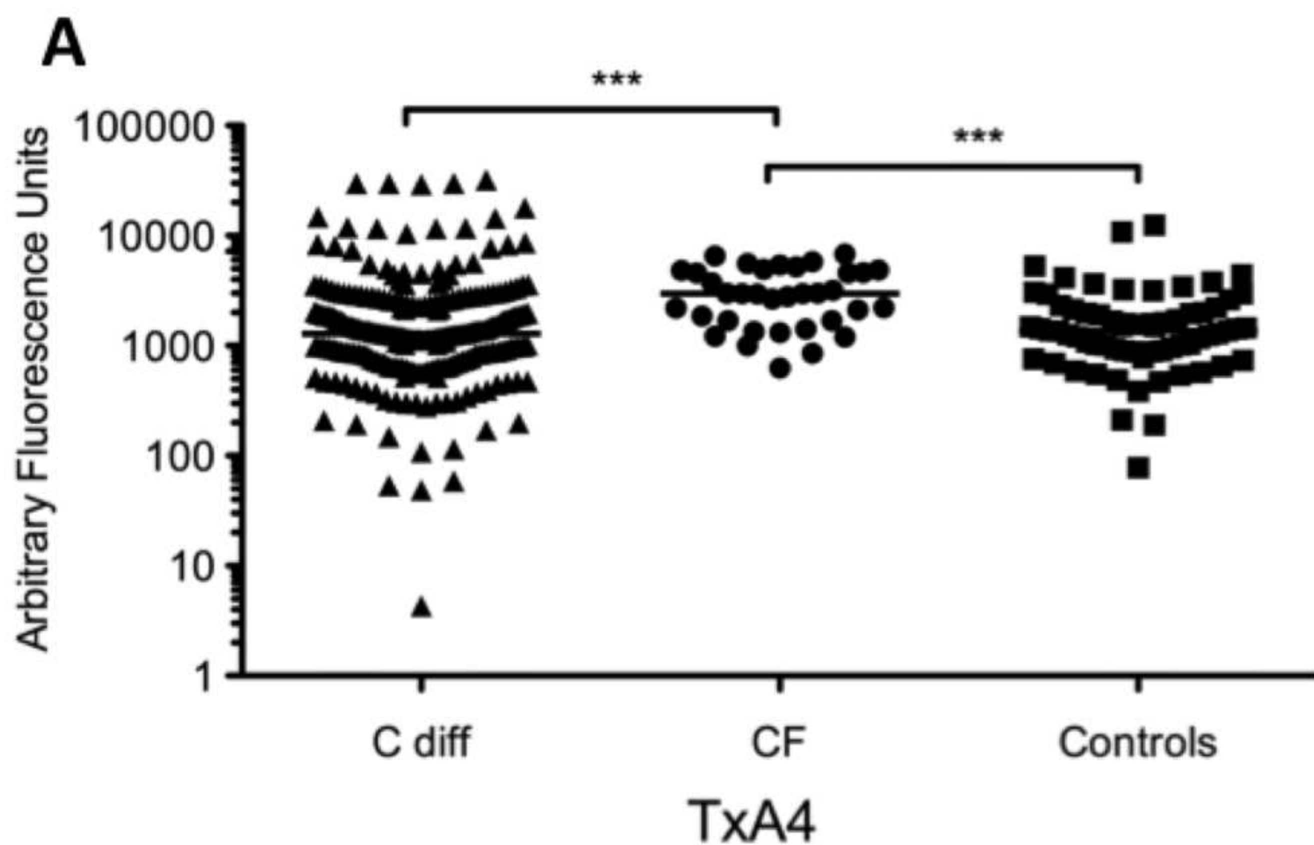

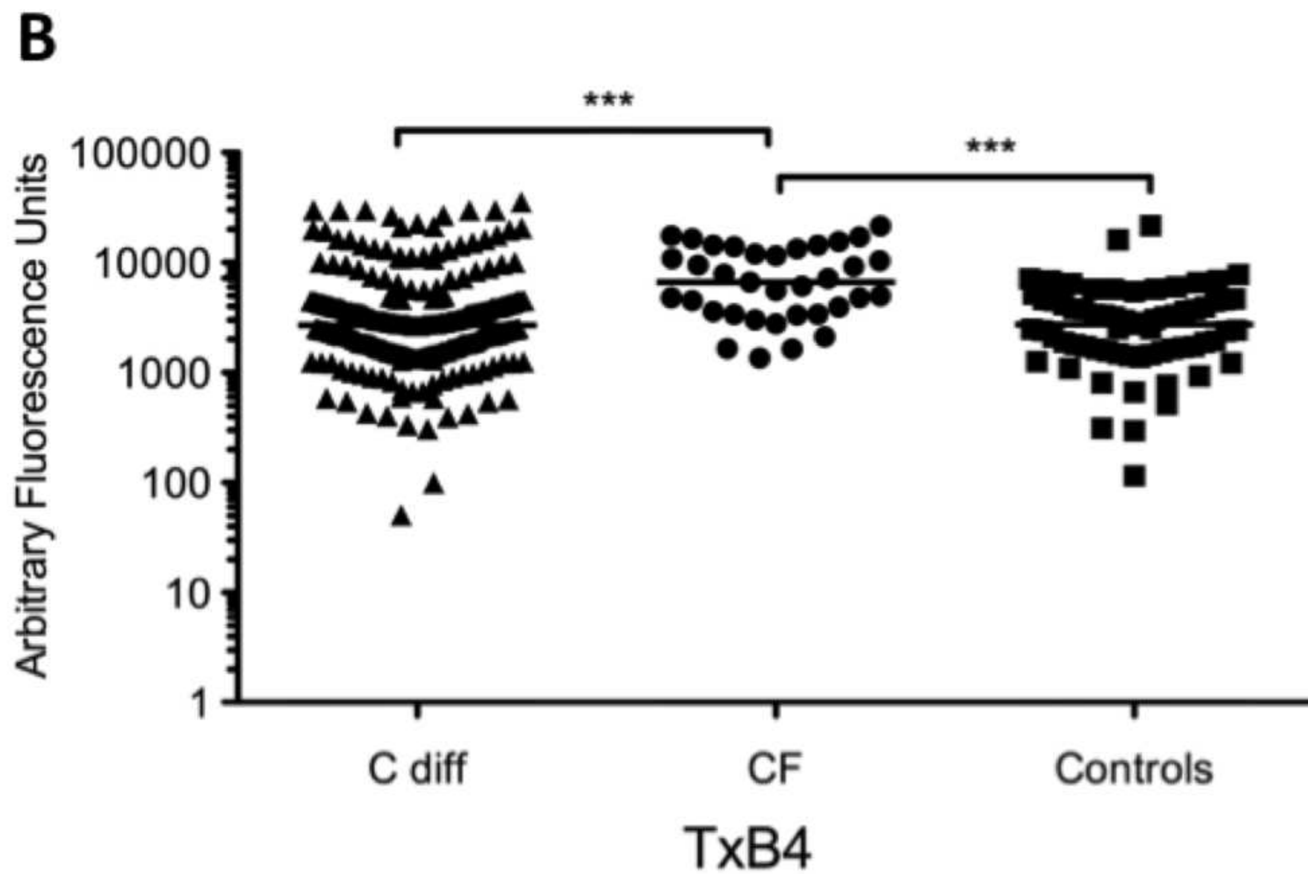

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