

**Title**

Protective antibodies against *Clostridium difficile* are present in intravenous immunoglobulin and are retained in humans following its administration

**Running Title**

Anti-*C. difficile* antibodies in IVIg

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## **Summary:**

The prevalence of serum antibodies against *C. difficile* (CD) toxins A and B in healthy populations have prompted interest in evaluating the therapeutic activity of intravenous immunoglobulin (IVIg) in individuals experiencing severe or recurrent *C. difficile* infection (CDI). Despite some promising case reports, a definitive clinical role for IVIg in CDI remains unclear. Contradictory results may be attributed to a lack of consensus regarding optimal dose, timing of administration and patient selection as well as variability in specific antibody content between commercial preparations. The purpose of this study was to retrospectively investigate the efficacy of three commercial preparations of IVIg for treating severe or recurrent CDI. In subsequent mechanistic studies using protein microarray and toxin neutralization assays, all IVIg preparations were analyzed for specific binding and neutralizing antibodies (NAb) to CD antigens *in vitro* and the presence of anti-toxin NAbs *in vivo* following IVIg infusion. A therapeutic response to IVIg was observed in 41% (10/17) of the CDI patients. Significant variability in multi-isotype specific antibodies to a 7-plex panel of CD antigens and toxin neutralization efficacies were observed between IVIg preparations and also in patient sera before and after IVIg administration. These results extend our current understanding of population immunity to CD and support the inclusion of surface layer proteins and binary toxin antigens in CD vaccines. Future strategies could enhance IVIg treatment response rates by using protein microarray to preselect donor plasma/serum with the highest

levels of anti-CD antibodies and/or anti-toxin neutralizing capacities prior to fractionation.

**Introduction:**

*Clostridium difficile* (CD) is the leading cause of hospital-acquired infective diarrhoea and a global health problem [1]. CD exerts its major pathological effects through two pro-inflammatory and cytotoxic protein exotoxins, A and B. Some strains also produce a third protein toxin known as binary toxin or CDT. Non-toxin virulence factors such as surface layer proteins (SLPs) also appear to be involved in pathogenesis [2-5]. Several clinical studies have previously reported associations between the ability to mount serum antitoxin antibody responses, favorable clinical outcome and the absence of CDI recurrence [6-10].

Early population prevalence studies also indicate that the majority of healthy adults have detectable antibodies to CD toxins A and B in their sera that are thought to arise from colonization in infancy or from repeated environmental exposure to CD in adulthood [11-12]. For this reason, polyclonal IVIg has been used off-label to treat both recurrent and fulminant *C. difficile* infection (CDI). Human intravenous immunoglobulin (IVIg) consists of purified plasma immunoglobulins from hundreds to thousands of healthy blood donors. Although several encouraging case reports highlight the potential benefits of IVIg, its definitive clinical role is still unclear, mainly due to the lack of robust evidence from randomized controlled trials [13-16]. Contradictory results obtained in respect to its clinical efficacy may be in part ascribed to the poor

characterization of commercial IVIg preparations in terms of their specific antibody content.

The mode of action of IVIg remains poorly understood. While some attention has focused on the varying capacity of IVIg to treat recurrent CDI, presumably by neutralizing CD toxins A and B [13], the full repertoire of CD-associated protein targets of these complex preparations remain ill-defined, as do the subclass distribution of these specific antibodies. Furthermore, the exact prevalence, kinetics and individual variation of binding and neutralizing antibodies (NAb) against CD proteins in serum samples, including those exposed to IVIg, are poorly described. Microarray assays are a promising new tool for compositional bioanalysis of specific antibody content in patient sera and IVIg due to their high sensitivity, reproducibility and ease of use. We have determined whether the IVIg products used in our institution possess specific binding and neutralizing antibodies to CD antigens *in vitro* and whether anti-toxin NAbs are present *in vivo* following IVIg infusion.

#### **Materials and Methods:**

**Patients and samples:** We retrospectively investigated the efficacy of three commercial preparations of IVIg (Vigam® BPL, Privigen® CSL Behring and Intratect® Biotest UK) in the treatment of adult patients with protracted, recurrent or severe CDI at Nottingham University Hospitals NHS Trust between 2012 and 2015. CDI cases (Cohort 1) were defined as patients with diarrhea (at least three loose stools per day for at least two consecutive days) and cytotoxin-positive feces. Medical records were reviewed for the following data: patient demographics, disease severity (ZAR score) [16, 17], previous CDI

treatment, IVIg type, timing (days from diarrhea to infusion), dosage and response to treatment, complications of IVIg therapy, need for colectomy, in-hospital mortality and CARDS risk of death score [18]. For Cohort 1, stored serum samples were not available for serological analysis. We therefore profiled sera from patients (Cohort 2) before and immediately after administration of IVIg treatment for combined immunodeficiency disorder [CVID; n=5 (47 (41-68 years)], chronic inflammatory demyelinating polyneuropathy (CIDP; n=1; 65 years of age) and CDI (n=1, 71 years of age). All subjects provided written informed consent under approvals granted by the Nottingham Research Ethics Committee.

**Antigen Microarray:** Binding of antibodies within IVIg preparations and patient sera to specific CD antigens were determined by using a previously validated CD protein microarray [19]. In brief, seven CD antigens, two positive controls: tetanus toxoid and lysates from *Candida albicans*, a negative control (printing buffer) and 10-point two-fold serial dilutions of human immunoglobulin (matching the tested antibody isotype) were spotted onto aminosilane slides (Schott, Germany) in quadruplicates using MicroGridII arrayer (Digilab, USA) and a silicon contact pin (Parallel Synthesis Technologies, USA). The seven CD antigens used in this study were: highly purified CD whole toxins A (200µg/mL) and B (100µg/mL; toxinotype 0, strain VPI 10463, ribotype 087), toxin B from a CD toxin-B only expressing strain (CCUG 20309; 90µg/mL), precursor form of B fragment of binary toxin, pCDTb, (200µg/mL; produced from a wholly synthetic recombinant gene construct; amino acid sequence based on published sequence from 027 ribotype <http://www.uniprot.org/uniprot/A8DS70>) and

purified native whole ribotype-specific (001, 002, 027) surface layer proteins (SLPs; all 200µg/mL). Multi-isotype (IgG, IgG1, IgG2, IgG3, IgG4, IgA, IgA1, IgA2 and IgM) antibody levels in serum samples and in IVIg preparations were tested against the CD panel of antigens. Slides were scanned at 635nm and the resultant images were processed with Genepix Pro-6 Microarray Image Analysis software (Molecular Devices Inc.). Protein signals were determined after background subtraction through customized modules in the R statistical language to generate general mean of signal levels. Specific isotype responses were interpolated against the internal isotype standard curve for each sample.

#### **Antibody Neutralization Assay:**

A Caco-2 cell-based assay for anti-toxin A and anti-toxin B NAb was used as previously published [20]. Briefly, Caco-2 cells (HTB-37; ATCC) were maintained in minimal essential medium (MEM) plus 20% fetal calf serum, 2mM glutamine and nonessential amino acids at 37°C. Serum samples were diluted in the assay medium at three dilutions (1:10, 1:100 and 1:1000). Then premixed with toxin A or toxin B (at 50% lethal dose [LD50]) for 1h at 37°C before 50µL of this mixture was transferred to the cells and incubated for 96 h. Following aspiration of the medium, 50µL methylene blue (0.5% [wt/vol] dissolved in 50% [vol/vol] ethanol) was added to the cell culture and incubated for 1h at room temperature. Then, the cells were washed gently with tap water (to remove excess stain) and air dried. The cells were then lysed by adding 100µL 1% (vol/vol) N-lauryl-sarcosine and incubated on a shaker for 15 minutes at room temperature. The cell biomass was determined by measuring the absorbance of each well on a BioTek Synergy2 (BioTeK, USA) plate reader at 405nm. Toxin

activity and working LD50 concentrations were defined empirically in preliminary experiments and for each individual batch/lot of toxin used.

### **Statistical analysis**

All statistical analyses were performed on natural log-transformed data using GraphPad Prism version 6 (GraphPad software, San Diego, CA). For non-paired data and multiple comparisons, the one-way ANOVA test was applied. For paired data, the Wilcoxon signed-rank test was used. Demographic data were presented as median and ranges. A p value  $\leq 0.05$  was deemed statistically significant.

### **Results:**

Before IVIg treatment, all patients in Cohort 1 had received high dose oral vancomycin (500mg four-times daily) and intravenous metronidazole (500mg four-times daily). All patients received 0.4g/kg of IVIg. Compared with non-responders [n=10/17; 75 (58-85) years], responders to IVIg [n=7/17; 82 (50-90) years] had lower ZAR disease severity [3(1-6) vs 5 (2-8), p= 0.14], CARDS risk of death scores [6 (3-15) vs 10.5 (2-14), p=3.1] and inpatient mortality [3 of 7 vs 7 of 10)], although these findings did not reach statistical significance. There were no statistically significant differences between the treatment response subgroups in the type of preparation, timing of administration or number of IVIg infusions received. No complications were reported for IVIg. Two patients underwent urgent colectomy in the non-responder group.

Specific antibody reactivities against CD proteins varied between the different commercial IVIg preparations as shown in the heatmap in figure 1A. Briefly, all IVIg preparations showed IgG reactivity to all tested CD antigens, although a



weaker response was observed to the SLPs. Vigam contained significantly higher levels of IgG1 antibodies against all toxins compared with Privigen and Intratect. Moreover, the antibody neutralization assay showed variability in percentage protection against CD toxins A and B between the different IVIg preparations. Here, Intratect at a 1:100 titration demonstrated a significantly lower protective capacity to neutralize CD toxin A compared with Vigam and Privigen (figure 1B).

For Cohort 2, the microarray data showed post-IVIg infusion enhancement in the levels of total IgG, IgG1, IgG2 and IgG3 to CD antigens (native toxins A and B, both VPI 10463), binary toxin (pCDTb) and toxin B (CCUG 20309), in all patients' sera (figure 2A). A statistically significant increase ( $p < 0.05$ ) was observed in the levels of total IgG against all toxins tested following IVIg administration (figure 2B). Notably, the highest IgG binding response was against toxin B ( $p = 0.0006$ , data not shown). However, there was no difference in post-IVIg NAb responses between toxins A and B ( $p = 0.0728$ , data not shown). For IgG1, this increase was significant against toxin B, binary toxin (pCDTb) and toxin B (CCUG 20309) only. Moreover, IgG2 antibody levels were significantly increased ( $p < 0.05$ ) against toxin B, and toxin B (CCUG 20309). Interestingly, following IVIg infusion, the level of IgG3 was increased against toxin A, toxin B, toxin B (CCUG 20309), and SLP027, but the magnitude was not statistically significant. Serum samples from all cohort 2 patients after IVIg infusion demonstrated significantly enhanced anti-toxin A and anti-toxin B antibody neutralization activities (figure 2C) at 1:10 dilution. However, the antitoxin NAb effect was reduced at higher serum dilutions (data not shown).

## **Discussion:**

Although no patients in either cohort experienced complications attributable to IVIg therapy, only 41% of the CDI patients in Cohort 1 showed a therapeutic response to IVIg with two patients requiring emergency colectomy for fulminant CDI in the non-responder group. These findings are in keeping with an earlier observational study by Abougergi *et al* [9] which revealed that 43% of patients survived their hospitalization with CDI colitis resolution following IVIg. These observations may reflect inadequate dosaging, delayed treatment, insufficient binding and/or neutralizing titers and more severe disease. Importantly, our findings do show the limited efficacy of Intratect in neutralizing toxin A and suggest that Vigam or Privigen may be the preferred IVIg preparation of choice for use in the CDI population.

We believe this is the first report that demonstrates the prevalence of CD anti-binary toxin and anti-SLP antibodies in all tested human IVIg preparations and in patient sera pre- and post IVIg treatment. Our data also confirm the detection of protective anti-toxin A and anti-toxin B NAbs in patient sera following treatment. Variability in specific antibody content between the different IVIg preparations examined in this study and that reported in an earlier study by Salcedo *et al* [1] may be due to the different geographical regions from which the plasma samples were collected and/or differences in CD exposure. Our binding data for IgG revealed significantly higher levels of anti-toxin B IgG in post IVIg sera. This finding seems to confirm the recent Merck monoclonal antibody Phase 3 trial which showed that an anti-toxin B response was the prime determinant for preventing CDI relapse [21]. Enhanced IgG2 and IgG3

immunoreactivities seen following IVIg infusion may prove highly beneficial given their more desirable molecular and functional attributes. Indeed, Katchar *et al* [22] detected humoral immune deficiencies in the IgG2 and IgG3 subclasses directed towards toxin A in patients with recurrent CDI. The lack of a post-IVIg IgG4 response is perhaps indicative that the immune response has not been pushed through to repeat antigen challenge. Differences in observed toxin neutralizing efficiencies might be caused by a combination of anti-toxin antibody titers, as well as by individual differences in toxin potencies. Interestingly, none of the CVID and CIDP patients receiving three weekly IVIg infusions in Cohort 2 had previously developed CDI. This may be because of protective anti-toxin NAb in the IVIg. Although most CD protein toxins should be neutralized by IVIg treatment, we were unable to study anti-binary neutralizing capacities within the IVIg or patient sera. Moreover, we did not examine antibody affinities for the CD antigens described in this report. Although there were no stored sera available for Cohort 1, we did compare binding and NAb pre- and post-infusion in a second mainly non-CDI cohort. It is noteworthy that the diarrheal symptoms of the CDI patient that received IVIg in Cohort 2 resolved within 4 days of IVIg (Privigen) infusion.

These results might be helpful for optimizing the type and dosage of IVIg used in adjunctive therapy for CDI, and further support a possible rationale for inclusion of SLPs and binary toxin antigens in future candidate CD vaccines. Further studies are required to measure antibody affinities and to clarify the precise contribution of different IgG subclasses to clinical protection or to disease pathogenesis. These studies could be achieved by purifying out the

IgG subclass-specific antibodies and assessing their significance (including their potential to interfere with or block the action of other IgG subclasses) in well-validated functional assays [23]. For example, an early study compared purified subclass antibodies in Herpes virus neutralization assays, determining that IgG3 and IgG4 had the greatest viral neutralizing ability despite not being the predominant subclass [24]. A further study of responses against human enterovirus 71 found that IgG1 and IgG2 fractions were the most effective at neutralization, and that IgG3 led to enhanced infection [25]. The knowledge obtained from IgG subclass studies, combined with a greater molecular understanding of IgG subclass properties will facilitate the engineering and development of highly effective CD-specific monoclonal therapeutic antibodies. Despite ongoing debate as to the utility of IVIg for CDI, future strategies could attempt to enhance the opportunities of this drug to show therapeutic efficacy and survival through application of disease severity risk scores, which should prompt earlier identification of those patients who are likely to require and receive most benefit from IVIg [17-18]. A review of the severe cases of CDI published in the medical literature suggests that the earlier administration of IVIg may increase the likelihood of attaining therapeutic efficacy and survival [16]. Moreover, given that the concentration and antimicrobial specificities of the antibodies are not normally routinely evaluated in batches of commercial polyclonal IVIg, donor units delivered to the fractionation sites that have high antibody levels against CD antigens could be identified using microarray technology and stored in biobanks. Donor plasma/serum could even be prescreened before donation to identify optimal batches with the highest levels

of CD-reactive IgG. Alternatively, the anti-CD activity of IVIg could be further enhanced by acquiring blood samples from patients convalescing from CDI or from vaccinated individuals. Such an enrichment strategy has been successfully used to treat viral diseases [26-28], and is regarded by WHO as a potential treatment for Ebola Virus Disease [29]. While the breadth of protection may still be limited by ribotype or strain-specific differences in protein expression, hyper-immune IVIg (H-IVIg) may represent a more effective adjunct for CDI than the polyspecific IVIg that is currently employed clinically. This therapy should be studied in a head to head comparison with polyclonal IVIg and antitoxin levels within patient sera should be correlated with clinical outcomes.

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Author contributions

ON, BM and OA performed the experiments. ON and MH transformed the data sets. CS, DH, and KA provided purified toxins and CL, IM and ML provided the surface layer proteins for the study. MH proof read and edited the manuscript. TM designed the study, recruited all subjects and wrote the manuscript.

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**Conflict of Interest:**

Ola H. Negm: No conflict

Brendon MacKenzie: No conflict

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Omar AJ. Ahmad: No conflict

Clifford C. Shone: No conflict

David P. Humphreys: owns UCB stock options.

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Mark Lynch: No conflict

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Tanya M. Monaghan: No conflict

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### Figure Legends:

**Figure 1: Immune reactivity and neutralizing effect of IVIg to *C. difficile* antigens.**

**A) Reactivity of multi-isotype specific antibodies to *C. difficile* antigens in commercial IVIg preparations:** Heatmap produced by Multiple Experiment Viewer (MeV 4.9) illustrates the levels of specific antibody isotypes (IgG, IgG1, IgG2, IgG3, IgG4, IgA, IgA1, IgA2 and IgM) in three commercially available IVIg preparations; Vigam, Privigen and Intratect, against seven *C. difficile* antigens [toxin A (200µg/mL, toxin B (100µg/mL), pCDTb (200µg/mL), toxin B (CCUG 20309; 90µg/ml) and surface layer proteins (SLPs) 001, 002, and 027; all 200µg/mL] using protein microarray technology. Colour code of the heatmap: green (low) to red (high) signal intensity. Signal values represented on the colour scale for the heatmap are log<sub>2</sub> transformed from the arbitrary fluorescence units (AFU). 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) IVIg neutralization efficacy against *C. difficile* native whole toxins toxin**

**A and toxin B:** Percentage protective neutralization effect of commercial IVIg products; Vigam, Privigen and Intratect against *C.difficile* toxins A and B. Each plot represents the median of triplicate experiments at 1:100 dilution. Intratect exhibits the lowest protective effect compared to Vigam and Privigen, particularly against toxin A. *P* values of \*\*\*\*  $\leq 0.0001$ ; \*  $\leq 0.05$  (one-way ANOVA).

**Figure 2: Immune reactivity and neutralizing effect of patients' sera to *C. difficile* antigens.**

**A) Comparison of antibody reactivities against *C. difficile* proteins in patients sera before and after IVIg infusion:** Heatmap produced by Multiple Experiment Viewer (MeV 4.9) illustrates the expression level of the isotypes (IgG, IgG1, IgG2, IgG3, IgG4, IgA, IgA1, IgA2 and IgM) in serum samples in 7 patients before and after IVIg infusion against seven *C. difficile* antigens [Toxin A (200µg/mL), Toxin B (100µg/mL), pCDTb (200µg/mL), Toxin B (CCUG 20309; 90µg/mL) and surface layer proteins (SLPs) 001, 002, and 027; all 200µg/mL] using protein microarray technology. Colour code of the heatmap: green (low) to red (high) signal intensity. Signal values represented on the colour scale for the heatmaps are log<sub>2</sub> transformed from the arbitrary fluorescence units (AFU). There was post infusion enhancement of the total IgG, IgG1, IgG2 and IgG3 reactivities to Toxin A, Toxin B, and pCDTb.

**B) IgG responses to toxins A, B and binary toxin (pCDTb) pre- and post-IVIg administration.** Pre- and post-IVIg IgG anti-toxin levels showing significant increase of total IgG against all toxins tested following IVIg infusion (Wilcoxon signed-rank test). Each plot represents the median of triplicate experiments at 1:10 dilution.

**C) Neutralisation effect against *C. difficile* native toxins A and B following IVIg administration:** Comparison of pre- and post-infusion neutralizing antibody activities showed enhanced protective effect after IVIg infusions against *C. difficile* native toxins (toxin A and toxin B). Each plot represents the median of triplicate experiments at 1:10 dilution. A significant increase in the

protective effect against toxin A and toxin B was noted in patient sera tested post IVIg infusion (Wilcoxon signed-rank test).