Human antibody response to surface layer proteins in *Clostridium difficile* infection

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Abstract

*Clostridium difficile* is a major cause of infectious diarrhoea in hospitalised patients. Surface layer proteins (SLPs) are the most abundant surface localised proteins expressed by *C. difficile*. The aim of this study was to examine the humoral immune response to *C. difficile* SLPs and its potential role in protection from *C. difficile* associated diarrhoea (CDAD). Serum antibodies to SLPs from *C. difficile* were measured by ELISA in a cohort of 146 patients (55 patients with CDAD, 34 asymptomatic carriers, and 57 controls). No significant difference was detected in serum IgM, IgA or IgG antibody levels between cases, carriers or control groups at any of the time points tested. However, patients with recurrent episodes of *C. difficile* diarrhoea had significantly lower IgM-anti-SLP levels than patients with a single episode on days 1, 3, 6 and 9 (\(p = 0.05, p = 0.009, p = 0.02, p = 0.049\)). The adjusted odds ratio for recurrent diarrhoea associated with a low day 3 serum IgM anti-SLP antibody level was 24.5 (95% confidence interval; 1.6–376.3). Further studies which examine the specific anti-SLP antibody responses to the colonising strain are warranted to determine if immune responses to *C. difficile* SLPs play a role in protection from CDAD.

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1. Introduction

*Clostridium difficile* is a common nosocomial pathogen and a major cause of infectious diarrhoea in hospitalised patients [1,2]. Antimicrobial therapy leads to an alteration of the normal colonic microflora, allowing this opportunistic pathogen to colonise. Patients receiving antibiotic therapy while in hospital are especially likely to be exposed to *C. difficile* and become infected [3]. Colonisation is associated with a wide spectrum of clinical presentations ranging from the asymptomatic carrier state to fulminant pseudomembranous colitis [4]. Pathogenic strains of *C. difficile* produce two potent exotoxins, toxin A and toxin B. The role of these toxins in the pathogenesis of *C. difficile* associated disease (CDAD) has been investigated extensively [5].

A number of clinical studies have highlighted the importance of the host immune response in *C. difficile* infection [6]. One recent study demonstrated a strong association between serum IgG anti-toxin A antibodies and protection against CDAD [3]. A second study, found that an IgG response to toxin A during an initial episode of CDAD was associated with protection from recurrent episodes of diarrhoea [7]. In addition to describing antibody responses to the well known *C. difficile* antigens, toxins A and B, antibody responses to non-toxin antigens were also evaluated [7]. Patients with recurrent CDAD were found to have significantly lower IgM antibodies against a crude preparation of non-toxin antigens...
antigens compared to patients with a single episode of diarrhoea [7]. These data suggest that the host immune response to *C. difficile* antigens, other than toxin A and B, may play an important role in disease expression.

Many bacterial species including *C. difficile* express a paracrystalline surface protein array, termed an S-layer, composed of one or two surface layer proteins (SLPs) [8,9]. SLPs have been previously described as virulence factors for *Aeromonas salmonicida* and *Campylobacter fetus* and as bacterial adhesins in *Lactobacillus acidophilus* [10–13]. SLPs are the predominant surface proteins in *C. difficile* and have been characterised recently at a molecular level [14,15]. They consist of two surface layer proteins derived from a single gene product. The higher molecular weight protein (MW 42–48 kDa) is highly conserved between strains, while the lower molecular weight protein (MW 32–38 kDa) demonstrates considerable sequence diversity [16,17].

Previous studies have demonstrated that the lower molecular weight *C. difficile* SLP contains antigenic epitopes [18–20]. The higher molecular weight protein has recently been described as an adhesin, involved in the adherence of *C. difficile* to human intestinal tissue [21].

The aims of this study were to determine if *C. difficile* SLPs were recognised as immunogens and whether the presence or magnitude of an antibody response correlated with the risk for colonisation and/or the clinical outcome of *C. difficile* infection in a cohort of hospital patients receiving antibiotics.

2. Materials and methods

2.1. Patient sera

Stored sera from previous prospective cohort studies of the host immune response to nosocomial *C. difficile* infection were examined [3,7]. These included sera from patients with CDAD (n = 55), from asymptomatic carriers of *C. difficile* (n = 34) and non-colonised controls (n = 57). Diarrhoea was defined as a change in bowel habit with 3 or more unformed bowel movements for at least 2 days. *C. difficile* diarrhoea was defined as diarrhoea not attributed to any other cause and associated with a positive stool test for *C. difficile* toxin. Recurrent CDAD was defined as the occurrence of a new episode of CDAD confirmed by a positive *C. difficile* stool toxin test, within a 60 day follow-up period, after resolution of the enrollment episode for at least 48 h and after discontinuation of therapy with metronidazole or oral vancomycin. Asymptomatic carriage was defined as a positive *C. difficile* stool culture or toxin test and the absence of diarrhoea during hospitalisation and during a 30 day monitoring period after discharge. Controls were hospitalised patients receiving antibiotic therapy who were not colonised with *C. difficile*. Additional information regarding clinical study design, patient demographics and risk factors for CDAD has been published previously [3,7,22].

2.2. Preparation of *C. difficile* surface layer proteins

Surface layer proteins were extracted from *C. difficile* isolates 101, 291, 371 and 959 (toxigenic *C. difficile* isolates obtained during the prospective cohort studies) as previously described [14,15,23]. Bacterial cells from a 24–72 h anaerobic culture in 50 ml of brain heart infusion broth (BHI) were harvested by centrifugation (15 min, 2700g), washed once in PBS, and re-suspended in 200 μl of 0.2 M glycine pH 2.2. After 30 min incubation at room temperature, the mixture was centrifuged to remove the bacteria, and the supernatant neutralised with Tris and saved for analysis [23]. The high and the low molecular weight subunits were separated by preparative SDS-PAGE (Fig. 1) [24]. SLP samples were loaded on 1.5-mm thick gels made to a final acrylamide concentration of 10% using an acrylamide:bis-acrylamide ratio of 30:0.8. Electrophoresis was run at constant voltage (120 V), until the bromophenol blue indicator reached the bottom of the gel, after which one side lane was cut out and stained with Comassie blue R-250 to identify protein bands. This lane was then realigned to the unstained portion of the gel, from which a slice corresponding to each of the SLP subunits was cut out, briefly rinsed in H2O and chopped into small fragments. Elution was carried out at 37 °C in ~1 volume of 10 mM sodium phosphate buffer pH 6.8, 0.1% SDS, replaced three times over 36 h. To remove the SDS, protein was precipitated by the addition of 4 volumes of cold acetone followed by incubation for >30 min at −20 °C [25]. The precipitate was recovered by centrifugation at 10,000g for 15 min, washed once with cold ace-

![Fig. 1. SDS-PAGE analysis of native, acid-extracted SLPs. SDS-PAGE analysis of native, acid-extracted SLPs (Tot) and purified high MW (Up) and low MW (Low) subunits from the *C. difficile* isolates (101, 291, 371, 959) used in this study. Samples were run on 12.5% gels. Staining was with Coomassie Blue R-250. The solid arrowheads point to high and low-MW subunits in all strains. The positions of molecular mass standards (in kilodaltons) are indicated on the right.](https://academic.oup.com/femspd/article-abstract/41/3/237/496227/Human-antibody-response-to-surface-layer-proteins/319x135)
Methanol:acetic acid:water (80:10:0.2:9.8), vacuum-dried and resuspended in 0.5 ml of 10 mM NH₄HCO₃.

3. Elisa

Microtiter plates were coated with SLPs by overnight incubation at 4 °C with 100 μl of C. difficile SLP, containing 1.3 μg ml⁻¹ of protein in carbonate–bicarbonate coating buffer (pH 9.6). After washing with PBS (pH 7.2)–0.05% Tween 20, blocking solution [2% (w/v) BSA, 0.05% (v/v) Tween 20] in PBS (200 μl) was added to each well and incubated for 1 h at room temperature. After further washing, serum samples diluted 1/50 in PBS–0.1% BSA were added and plates were incubated for 1 h at room temperature. After washing, 100 μl of horseradish peroxidase-conjugated rabbit anti-human class-specific IgM, IgG or IgA, diluted 1/1000 in PBS–0.1% BSA was added and incubated for 1 h at room temperature. The ELISA was developed using TMB (3,3′,5,5′-tetramethyl benzidine) microwell peroxidase substrate system (Kirkegaard and Perry Labs, Gaithersburg, MD, USA). The reaction was stopped after 2 min by the addition of 100 μl 1M phosphoric acid. Optical density (OD) was measured at a wavelength of 450 nm with a reference filter of 630 nm using a microELISA plate reader (Dynatech MR600). Antibody levels were reported as mean optical density of duplicate samples.

3.1. Statistical analysis

Antibody measurements using SLP-291 as the capture antigen were normally distributed; therefore we used analysis of variance and Student’s t tests to test the null hypothesis that there was no difference in mean antibody levels between (i) non-colonised patients, patients with CDAD and asymptomatic carriers and (ii) patients with recurrent and non-recurrent diarrhoea. We examined associations between mean antibody levels and other variables (age, use of additional antibiotics, disease severity and other antibody levels at other time points) using Student’s t tests, analysis of variance or Pearson’s correlation coefficients. We separated antibody levels in patients with C. difficile diarrhoea into quartiles on the basis of the distribution of antibody levels in serum samples drawn at the onset of diarrhoea. Using the Mantel–Haenszel χ² test we then determined whether there was a trend associated with decreasing antibody levels and risk of recurrent diarrhoea. We examined the association of recurrent diarrhoea with the highest quartile of antibody level (high) versus the lower three quartiles (low-normal) with two-tailed Fisher’s exact tests. We used multivariable logistic regression analysis to determine the adjusted odds ratio for recurrent C. difficile diarrhoea associated with low-normal antibody levels, while controlling for age, use of additional antibiotics and disease severity. Analyses were performed using the SAS software system, version 6.12 (SAS institute Inc, Carey, NC, USA). The α level was set at 0.05. All P values were two-sided.

4. Results

4.1. Antibody response to colonising C. difficile strains

We initially examined serum antibody responses to the SLPs (high and low MW SLPs combined, SLP high alone and SLP low alone), from four clinical isolates of C. difficile obtained from patients in our study cohort. Two of these isolates (101 and 371) were obtained from patients with CDAD, while two (291 and 959) were from asymptomatic carriers. For each subject we measured serum antibody levels to the specific SLPs purified from their colonising strain, thus allowing us to determine strain specific anti-SLP responses. These studies indicated a trend, whereby patients who carried the organism but remained asymptomatic had higher IgM antibody levels to the SLPs than patients who went on to develop CDAD (Fig. 2).

4.2. Antibody response to SLPs from isolate 291 in all patients

We then extended our study to examine 450 sera obtained from 146 patients in our study cohort during the course of their hospitalisation. Patients were categorised into cases, carriers and control subjects. We examined...
IgM, IgG and IgA antibody levels to SLP isolated from strain 291 (SLP-291) in sera collected at the time of hospital admission and at the time of \textit{C. difficile} colonization (or the mid-point of hospital stay for controls). There were no significant differences in IgG, IgM or IgA anti-SLP-291 antibody levels measured at either of these time points. When the total patient population was examined (\(n = 146\)), marked variation in antibody levels were found for all antibody subtypes with ODs ranging from 0.09 to 1.81 for IgM, 0.2 to 2.2 for IgG and 0.07 to 1.9 for IgA (data not shown). IgG anti-SLP levels were positively correlated with IgA anti-SLP levels at the time of \textit{C. difficile} colonisation (\(R = 0.42, p < 0.001\)). There was no significant correlation between IgM and IgG antibody levels or IgM and IgA antibody levels at this time point.

4.3. Antibody response to SLPs from isolate 291 in patients with CDAD

We then examined IgM, IgG and IgA anti-SLP antibody levels in sera collected from patients with CDAD. Serum antibody levels to SLP were measured in sera taken from patients on admission to hospital, on the first day of \textit{C. difficile} diarrhoea and every three days thereafter until 12 days after the onset of diarrhoea. On day 0, at the time of hospital admission and before the onset of \textit{C. difficile} diarrhoea, there was not a significant difference between patients who later developed recurrent CDAD compared to those who experienced a single episode. By day 3 however, mean serum IgM anti-SLP antibody levels were significantly higher in patients with a single episode of CDAD compared to patients with recurrent disease (Fig. 3). This difference in IgM antibody level remained significant through days 6 and 9 (Fig. 4). The levels of IgG and IgA anti-SLP were also compared for the two patient groups and no statistically significant difference was identified at any of the time points (Fig. 3 and data not shown).

4.4. Association between day 3 IgM anti-SLP antibody and recurrent CDAD

Antibody levels in patients with \textit{C. difficile} diarrhoea were categorised into quartiles on the basis of the distribution of antibody levels in serum samples drawn at the onset of diarrhoea. Patients were categorised as having a low IgM response if their anti-SLP level was less than the cut-off for the uppermost quartile (corresponding to an OD reading of less than 0.54). Those with anti-SLP levels in the range of the uppermost quartile were categorised as having a high IgM response (corresponding to an OD reading of greater than or equal to 0.54). Sixty-two percent of patients with a low IgM anti-SLP had one or more recurrent episodes of \textit{C. difficile} diarrhoea, while only 10% of patients with a high IgM anti-SLP had recurrent diarrhoea (Table 1).

The unadjusted odds ratio for recurrent diarrhoea associated with a day 3 IgM anti-SLP response of less than 0.54 was 15.0 (95% CI 1.6–138.8; \(p = 0.008\)). The odds
Table 1

<table>
<thead>
<tr>
<th></th>
<th>Single episode (n = 18)</th>
<th>Recurrence (n = 16)</th>
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<tbody>
<tr>
<td>Low-normal IgM anti-SLP (OD &lt;0.54)</td>
<td>9 (38%)</td>
<td>15 (62%)</td>
</tr>
<tr>
<td>High IgM anti-SLP (OD ≥0.54)</td>
<td>9 (90%)</td>
<td>1 (10%)</td>
</tr>
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ratio was then adjusted for age, disease severity, and antibiotic use during the follow up period, as these criteria were previously found to be independent risk factors for recurrent *C. difficile* diarrhea [22]. The adjusted odds ratio for recurrent diarrhea associated with a day 3 IgM anti-SLP response of less than 0.54 was 24.5 (95% CI 1.6–376.3; p = 0.02). Serum IgM anti-SLP antibody levels were significantly correlated to serum IgM antibody levels purified toxin A (R = 0.63, p < 0.0001) and against purified toxin B (R = 0.42, p = 0.01), three days after *C. difficile* diarrhea.

5. Discussion

Previous studies have demonstrated the role that bacterial SLPs play in pathogenesis of other gastrointestinal infections. As S-layers are the predominant surface protein in *C. difficile*, and may play an important role in *C. difficile* colonization and disease, our study examined antibody response to SLPs throughout the course of clinical infection in humans. Antibodies against SLPs obtained from a number of different *C. difficile* isolates demonstrated that SLPs are highly immunogenic. We confirmed that human sera contain detectable levels of antibodies to *C. difficile* surface layer proteins. Moreover, a considerable degree of variation among levels of antibody responses in different individuals was found for each of the three antibody subtypes investigated. We found that patients who had a single episode of *C. difficile* diarrhoea had significantly higher IgM antibodies to SLP at days 3 (p = 0.009) through 9 of their primary episode, than patients who later developed recurrent *C. difficile* diarrhoea. We also determined that a low IgM anti SLP level (<0.54) on day three of a primary *C. difficile* diarrhoeal episode was an independent predictor of recurrence, with a 25-fold increased risk of recurrent diarrhoea.

In the first instance we examined antibody response to total, upper and lower MW SLP isolated from colonising strains from four patients, thus allowing us to determine strain specific antibody response to SLP. Our data showed a trend where patients who carried the organism but remained asymptomatic had higher IgM antibody levels to the SLPs than patients who went on to develop CDAD. This finding suggested that strain specific antibody response to SLPs might be important for the clinical outcome of infection. Serum IgG antibody levels were also measured against strain specific *C. difficile* SLPs, however there were no apparent differences between cases and carriers for this antibody class. Three of the initial four *C. difficile* isolates were the same ribotype (ribotype 53) [26]. The fourth isolate (371) was ribotype 1. Unfortunately, other *C. difficile* strains from the study cohort were not available for testing, making it impossible to determine the frequency of each *C. difficile* ribotype.

For the large-scale assay of anti-SLP titers in our cohort of patients we have used a single arbitrarily chosen SLP type (from strain 291) rather than the specific type of the immunizing/colonizing strain(s) from each patient. There was no significant difference in antibody levels between controls, carriers or cases of *C. difficile* disease at any of the time points investigated. This may reflect the fact that we used only one antigen type (SLP-291) in this arm of the study and therefore we may not be measuring antibody response to the colonising *C. difficile* strain in all subjects. However, use of a single SLP is unlikely to have resulted in a significant underestimation of titers in some patients. Whilst the SLP low MW subunits are highly divergent in amino acid sequence, with homology typically lower than 50%, and show very limited immunological cross-reactivity, high MW subunits are highly (>68%) conserved both at the DNA and at the amino acid level, and cross-react extensively [16,17,27]. Moreover, there is at present no data to suggest that specific SLP types are associated with infection outcomes. Thus, any underestimation of antisera titers against specific SLP types is unlikely to cause a bias in the comparison between patient classes.

Previous studies by us and by others indicate that an inadequate immune response to *C. difficile* toxins, especially toxin A, is associated with a predisposition to recurrent CDAD, while an adequate anti-toxin antibody response is associated with protection [3,7]. Our present data suggest that immune response to other antigens may also play an important role in the outcome of *C. difficile* infection. The exact role played by SLPs in *C. difficile* colonisation, persistence of infection and development of CDAD remains unclear and further studies are needed to clarify these issues. Several other candidate antigens have now been characterised, including cell wall associated proteins that are related to the SLP [14,15,28]. The role of anti-SLP antibodies in protective immunity could also be investigated using an in vivo vaccination and challenge model of *C. difficile* infection [29,30].

In summary, we find that high IgM antibody levels to *C. difficile* SLPs are associated with a markedly reduced risk of recurrent *C. difficile*-associate diarrhoea. This finding is consistent with an important role for
C. difficile SLPs in intestinal colonisation, maintenance of infection and host protective immune responses. However, further studies are needed in individuals exposed to C. difficile to elucidate the effects of strain-specific immune responses to surface antigens on C. difficile colonisation and disease.

References