Mortality in patients with *Clostridium difficile* infection correlates with host pro-inflammatory and humoral immune responses

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Host anti-toxin immune responses play important roles in *Clostridium difficile* disease and outcome. The relationship between host immunity and inflammatory responses during severe *C. difficile* infection (CDI) and the risk of mortality has yet to be defined. We aimed to investigate the host systemic IgG anti-toxin immune responses, the *in vitro* cytotoxicity of the infecting *C. difficile* ribotyped strain, and the host inflammatory markers and their relationship to CDI disease severity and risk of mortality. Inflammatory markers, co-morbidities and CDI outcomes were recorded in a prospective cohort of 150 CDI cases. Serum anti-cytotoxin A (TcdA) and anti-TcdB IgG titres were measured by ELISA and the infecting *C. difficile* isolate was ribotyped and the *in vitro* cytotoxin titre assessed. A low median anti-TcdA IgG titre was significantly associated with 30-day all-cause mortality (*P*, 0.05). Ribotype 027 isolates were significantly more toxinogenic (*P*, 0.00001). High cytotoxin titres correlated with increased inflammatory markers but also higher anti-TcdA and -TcdB (*P*, 0.05) IgG responses resulting in a lower risk of mortality. On multivariate analysis, predictors of mortality were peak white cell count (odds ratio (OR) 11.53; 95 % confidence interval (CI) 2.38–55.92), creatinine concentration (OR 6.54; 95 % CI 1.47–29.07), Horn’s index (OR 4.09; 95 % CI 0.76–22.18) and low anti-TcdA IgG (OR 0.97; 95 % CI 0.95–0.99), but not ribotype, cytotoxin titre or anti-TcdB IgG. Thus, host pro-inflammatory and humoral responses correlate with the cytotoxin titre of the infecting strain and effective anti-toxin immune responses reduce the risk of mortality.

**INTRODUCTION**

*C. difficile* infection (CDI) is the most common cause of antibiotic-associated diarrhoea and colitis and is associated with a wide range of symptoms, from mild diarrhoea to fulminant colitis and death. Other risk factors, in addition to antimicrobial exposure, that are implicated in the development of CDI include advancing age (>65 years), prolonged hospital stay and severity of underlying disease (Cohen *et al.*, 2010; Kyne *et al.*, 2000; Pépin *et al.*, 2004; Thibault *et al.*, 1991). The host anti-toxin immunoglobulin response has been shown to play an important role in mediating the outcome of colonization with *C. difficile* (Kyne *et al.*, 2000), influencing the duration of disease (Warny *et al.*, 1994) and determining the risk of recurrence (Kyne *et al.*, 2001; Warny *et al.*, 1994). High serum anti-toxin IgG has demonstrated a degree of protection from severe, systemic CDI in animals (Johnson, 2012; Steele *et al.*, 2012), but the direct relationship between the host anti-toxin immunoglobulin response in severe CDI and protection from fatal outcome has yet to be defined.

Cases of severe CDI are increasing, reportedly associated with the emergence of ribotype 027/NAP-1/BI strain. Ribotype 027 strains are considered more virulent due in
part to hyper-production of cytotoxin A (TcdA) and B (TcdB) (Kuehne et al., 2010; Thelestam & Chaves-Olarte, 2000). High cytotoxin levels have been shown in vitro to elicit increased intestinal cell damage and provoke inflammatory responses. In vivo, severe CDI is characterized by marked leukocytosis [white cell count (WCC) \( >20 \times 10^9 \text{cells l}^{-1} \)], hypoaalbuminaemia (serum albumin <35 g l\(^{-1}\)) and elevated C-reactive protein (CRP; >40 mg l\(^{-1}\)) (Bauer et al., 2009). Complications of severe CDI include renal failure, sepsis, organ failure and death (McDonald et al., 2005), and are suggested to be due to dissemination of \( C. \) \( \text{difficile} \) TcdA and TcdB into the systemic circulation (Johnson, 2012; Steele et al., 2012).

The association between ribotype 027 strain type and CDI severity under non-outbreak conditions is controversial (Cloud et al., 2009; Wilson et al., 2010); therefore, the relationship between strain type, disease severity and cytotoxin production remains to be determined.

We conducted a prospective cohort study of CDI cases to investigate host systemic IgG anti-toxin immune responses and the relationship to CDI disease severity and risk of mortality. We also investigated the role of in vitro cytotoxicity of the infecting \( C. \) \( \text{difficile} \) strain in influencing host inflammatory and immune responses.

**METHODS**

**Patient clinical details and sample collection.** One hundred and fifty consecutive patients with CDI [positive stool test using Meridian ImmunoCard Toxins A/B (Meridian BioScience) and diarrhoea not attributable to any other cause] were recruited during 2008 and 2009 at two hospitals in Dublin, Ireland.

This study was approved by the local research ethics committees and consent was obtained from all participants. Patient demographic, clinical and laboratory data were collected, including maximum total peripheral WCC (\( \times 10^9 \text{l}^{-1} \)), serum CRP (mg l\(^{-1}\)), minimum serum albumin (g l\(^{-1}\)), peak serum creatinine level (\( \mu \text{mol l}^{-1} \)), Charlson morbidity scores and Horn’s index of severity of admitting diagnosis (Charlson et al., 1987; Horn et al., 1983). The most extreme value for these markers in the 72 h following the positive stool \( C. \) \( \text{difficile} \) toxin test was used for analysis.

The primary outcome was all-cause 30-day mortality rate. Secondary outcomes were recurrence \( \leq 60 \) days after resolution of primary diarrhoeal episode or mortality directly attributable to CDI as determined from death certificate data. It was also noted where CDI was recorded as a contributory but not the leading cause of death.

Stool samples were collected at diagnosis and \( C. \) \( \text{difficile} \) was isolated after alcohol shock and anaerobic culture on cycloserine-cefoxitin fructose selective agar (BD Biosciences) at 37 °C for 48 h.

Venous blood (10 ml) was drawn on days 1, 3 and 12 post-diagnosis into an anticoagulant-free vacuum tube and immediately centrifuged at 3000 g for 5 min. Serum was stored at \(-80 \) °C.

**\( C. \) \( \text{difficile} \) strain typing.** Genomic DNA was purified from \( C. \) \( \text{difficile} \) after overnight anaerobic growth in brain–heart infusion (BHI) broth (Oxoid), supplemented with 1% yeast extract and 0.1% (w/v) l-cysteine (Sigma-Aldrich) at 37 °C. DNA was extracted using a Wizard Genomic DNA Purification kit (Promega) and assessed for concentration and purity using a Nanodrop 2000 (Thermo Scientific) before use in PCR.

Isolates were characterized by PCR ribotyping as described previously (Stubbs et al., 1999) and ribotypes were assigned by comparison with representatives from the Leiden University Medical Centre library, Leiden, The Netherlands. Isolates were toxigenotyped as described previously (Rupnik et al., 1998). The presence of virulence factor-specific genes, encoding binary toxin (cdtB) and the 18/39 bp deletion in the regulatory gene tcdC, known to be associated with particular ribotypes, was also assessed by molecular methods, as described previously (Cohen et al., 2000).

**Quantification of anti-TcdA and -TcdB IgG concentration.** Levels of anti-TcdA and -TcdB IgG in patient sera were measured by direct ELISA using immobilized TcdA or TcdB proteins (provided by C. P. Kelly, Harvard Medical School, MA, USA) by a previously published method (Kyne et al., 2000).

The relative antibody concentration in arbitrary ELISA units (EU) for each sample was defined by extrapolation from an internal control standard curve (absorbance versus dilution factor) generated from pooled unused sera of arbitrary high, medium and low antibody levels. Peak anti-TcdA and -TcdB IgG concentrations within 12 days of CDI diagnosis were calculated for each patient.

**Evaluation of bacterial cytotoxin titre.** Bacterial isolates were grown overnight (16–18 h) in BHI broth supplemented with 1% yeast extract and 0.1% l-cysteine at 37 °C under anaerobic conditions to an optical density at 600 nm of 2.0. Broth culture supernatants were passed through a 0.2 \( \mu \)m filter and tenfold serially diluted (10\(^{1}–10^{8}\)-fold dilutions) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS (Sigma-Aldrich) and 1% (v/v) l-glutamine/penicillin/streptomycin solution (final concentrations of 2 mM l-glutamine, 100 U penicillin ml\(^{-1}\) and 100 \( \mu \)g streptomycin ml\(^{-1}\); Sigma-Aldrich). The broth dilutions were then applied in triplicate to confluent Vero (African green monkey kidney) cell monolayers (LG2 Standards) and incubated for 24 h at 37 °C in 5% CO\(_2\). The Vero cell monolayers were then assessed by inverted light microscopy (Nikon; Unitech) for cell rounding. The cytotoxin titre was defined as the log\(_{10}\) value of the broth supernatant dilution factor that was able to induce 50% cell rounding (10\(^{1}–10^{8}\)-fold dilutions; cytotoxin titre 1–8) as described in a previously published method (Kamiya et al., 1989).

Broth supernatants that did not induce cell rounding were classified as cytotoxin titre 0. \( C. \) \( \text{difficile} \) toxin specificity was confirmed by neutralization with \( Clostridium \) \( sordellii \) anti-toxin (\( C. \) \( \text{difficile} \) Toxin/ Antitoxin kit; TechLab).

**Statistical analysis.** To assess whether the median anti-TcdA or -TcdB IgG titres were significantly different in cases of recurrence versus non-recurrence or high versus low bacterial cytotoxin titre, the Wilcoxon rank-sum test was used. Student’s \( t \)-test was used to compare mean cytotoxin titres between ribotype 027 and non-ribotype 027 isolates. Correlations between cytotoxin titre and clinical markers of inflammation were analysed using Pearson’s correlation coefficient (\( r \)). Correlations between cytotoxin titre and outcome frequencies were analysed using the point-biserial correlation coefficient (\( r_{pb} \)).

Univariate analysis of the risk factors for 30-day all-cause mortality were carried out using Student’s \( t \)-test for parametric means, the Wilcoxon rank-sum test for non-parametric medians and a \( \chi^2 \) Fisher’s exact test for categorical outcome frequencies.

Multivariable logistic regression analysis was used to determine the independent predictors of mortality. \( P \) values <0.05 were considered...
significant. Analyses were performed using the SPSS (IBM) and SAS (SAS Institute Inc.) statistical packages.

RESULTS

C. difficile isolation and molecular typing

Stool samples were collected from 128 of 150 patients and C. difficile was isolated from the stool and assigned a ribotype and toxinotype for 93 patients. A fully characterized C. difficile strain, full clinical data (co-morbidity scores, laboratory markers of inflammation and CDI outcome) and at least two serum samples within 12 days post-diagnosis were available for 86 patients (mean age 74.5, range 35–97 years), who comprised our final study population.

Ribotype 106 was the most prevalent type isolated from our study population (36 %), followed by ribotype 174 (13.9 %), ribotype 078 (11.6 %) and ribotype 027 (9.3 %). The binary toxin gene (cdtB) was detected in 32.5 % of isolates, including all ribotype 027 and 078 isolates. A mutated tcdC gene was detected in 25.5 % of isolates; 9.3 % (n=8) contained an 18 bp deletion and a deletion at position 117 (all ribotype 027 isolates) and 16.2 % (n=14) contained a 39 bp deletion (isolates of ribotype 078 and 015).

Ribotype 027 isolates were identified as either toxinotype IIIa (n=6) or toxinotype IIIb (n=2). Ribotype 078 and ribotype 017 isolates were identified as type V and type VIII, respectively. All other toxinotyped isolates were identified as type 0.

Host serum anti-TcdA and -TcdB IgG response

Of the patients infected with a toxigenic C. difficile strain, nine (10.7 %) did not have detectable serum anti-TcdA IgG and four (4.8 %) did not have detectable anti-TcdB IgG within the 12 days after CDI diagnosis.

Patients infected with TcdA−/TcdB+, toxinotype VIII, ribotype 017 isolates (n=4) produced both anti-TcdB (median 13.2 EU) and anti-TcdA IgG (median 77.7 EU).

The relationship between patient age and level of anti-TcdA and -TcdB IgG response was assessed and no difference in mean age was found between patients exhibiting low and high serum anti-TcdA IgG titres (first quartile, 74.2 years, vs fourth quartile, 74.9 years) or between patients with low and high serum anti-TcdB IgG titres (first quartile, 74.7 years, and fourth quartile, 73.4 years).

Relationship between host serum anti-TcdA and -TcdB IgG response and outcome

The overall recurrence and 30-day all-cause mortality rates were 18.6 and 16.3 %, respectively. Of the 14 patients who died by day 30, death was directly attributable to CDI for seven patients (8.1 % overall) and was recorded as a contributory factor for five patients (5.8 % overall). The remaining two patients died of complications from an additional infection or after gastrointestinal surgery.

Patients who died within 30 days of CDI diagnosis were significantly more likely to have a low median anti-TcdA IgG titre than patients who were alive by day 30 [4.2 EU (range 0–86), interquartile range (IQR) 12.1, versus 25.2 EU (range 0–176.4), IQR 62.7; P<0.05]. Patients who died within 30 days of diagnosis had a lower median anti-TcdB IgG titre than patients who were alive by day 30 [7.8 EU (range 0–56.6), IQR 49.2, versus 18.0 EU (range 0–159.3), IQR 75.0]. However, this was not significant (P=0.07).

Patients who had a recurrence within 60 days of CDI diagnosis had no significant difference in median anti-TcdA IgG titre than patients who did not have a recurrence [31 EU (range 0.8–176.4), IQR 81.8, versus 16.1 EU (range 0–123.2), IQR 54]. There was also no significant difference in anti-TcdB IgG titre between patients with and without recurrence [14.7 EU (range 4.6–107.1), IQR 62.3, versus 18.0 EU (range 0–159.3), IQR 55.7]. The number of patients for whom death could be attributed to CDI was too low for statistical comparison.

Determination of bacterial cytotoxin titre

The majority of isolates in this study were toxigenic and induced a cytopathic effect, apart from two isolates (ribotype 010 and 001) that did not generate amplicons after toxinotyping PCR.

Isolates were grouped according to ribotype, and the mean cytotoxin titre was calculated for the most prevalent ribotype groups (n≥3). Ribotype 027 isolates exhibited the highest mean cytotoxin titre (4.75±0.42). Isolates of ribotypes 014, 015, 106, 174 and 001 exhibited mean cytotoxin titres >2. The ribotypes exhibiting the lowest mean cytotoxin titre were ribotypes 017 and 078 (2.8±0.82 and 1.7±0.67, respectively) (Fig. 1a). Ribotype 027 isolates demonstrated a higher mean cytotoxin titre than all other non-ribotype 027 isolates (4.75±0.42 vs 2.46±0.83; P<0.00001) (Fig. 1b).

To analyse the association between bacterial cytotoxin titre and host anti-toxin immune responses, we classified patients into two categories according to whether they were infected with a strain demonstrating a low (titre ≤3) or high (titre 4 or 5) cytotoxin titre. The majority of patients studied (n=75; 87 %) were infected with a C. difficile strain exhibiting a low in vitro cytotoxin titre.

Association between bacterial cytotoxin titre and anti-TcdA and -TcdB IgG concentration

Median concentrations of serum anti-TcdA IgG were significantly higher in patients infected with a high cytotoxin strain compared with those infected with a low cytotoxin strain [56.9 EU (range 8.4–123.2), IQR 47.8,
Median concentrations of serum anti-TcdB IgG were also significantly higher in patients infected with high cytotoxin strains compared with low cytotoxin strains [66.0 EU (range 8.9–159.3), IQR, 83.1 vs 15.2 EU (range 0–138.1), IQR 53.7; \( P < 0.05 \)].

**Correlation between bacterial cytotoxin titre and clinical inflammatory markers**

To investigate whether there was a correlation between the *in vitro* cytotoxin titre of the infecting bacterial strain and the severity of the host inflammatory response during infection, patients were grouped according to the cytotoxin titre of the infecting isolate and mean clinical markers of inflammation were calculated (Table 1).

All CDI patients in this study demonstrated hypoalbuminaemia indicated by serum albumin levels below 3.9 g dl\(^{-1}\).

There was a significant positive correlation between bacterial cytotoxin titre and CRP level \( (r=0.28; P<0.05) \), peak WCC \( (r=0.183; P<0.05) \) and minimum albumin concentration \( (r=0.214; P<0.05) \), indicating that a rise in the *in vitro* cytotoxin titre of the infecting strain was associated with an increase in patient inflammatory responses. Patients in the highest cytotoxin titre group (cytotoxin titre 5) presented with inflammatory markers versus 15.9 EU (range 0–176.4), IQR 52.7; \( P<0.05 \) (Fig. 2).

![Graph showing correlation between bacterial cytotoxin titre and clinical inflammatory markers](image_url)
Table 1. Correlation between in vitro bacterial cytotoxin titre of the infecting strain and host inflammatory markers

Cytotoxin titres are shown as mean values, with SD in parentheses. For ribotype, the number of isolates for each ribotype is shown in parentheses. The number of isolates is shown for each titre value (total n=86).

<table>
<thead>
<tr>
<th>Marker</th>
<th>In vitro cytotoxin titre</th>
<th>Total (n=86)</th>
<th>r</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 (n=2)</td>
<td>1 (n=7)</td>
<td>2 (n=24)</td>
<td>3 (n=42)</td>
</tr>
<tr>
<td>Maximum CRP (mg l⁻¹)</td>
<td>–</td>
<td>80.77 (30.07)</td>
<td>109.48 (98.92)</td>
<td>105.97 (66.25)</td>
</tr>
<tr>
<td>Minimum serum albumin (g dl⁻¹)</td>
<td>1.5 (0.7)</td>
<td>2.4 (0.97)</td>
<td>2.32 (0.5)</td>
<td>2.16 (0.47)</td>
</tr>
<tr>
<td>Peak WCC (× 10⁶ l⁻¹)</td>
<td>12.95 (6.43)</td>
<td>13.68 (5.36)</td>
<td>17.44 (9.29)</td>
<td>16.2 (9.58)</td>
</tr>
<tr>
<td>Peak creatinine (µmol l⁻¹)</td>
<td>162 (123.04)</td>
<td>142 (54.91)</td>
<td>104.8 (100.49)</td>
<td>152.1 (167.91)</td>
</tr>
<tr>
<td>Ribotype (n)</td>
<td>001 (1), 010 (1)</td>
<td>078 (4), 016 (2), 017 (1)</td>
<td>106 (7), 174 (6)</td>
<td>106 (22), 174 (6)</td>
</tr>
</tbody>
</table>

*P<0.05.
†Including two isolates of toxigenic type IIIb.

Risk factors for 30-day all-cause mortality

Significant risk factors associated with 30-day all-cause mortality were identified using univariate analysis. The three independent predictors of mortality were peak WCC concentration (>20×10⁹ cells l⁻¹), high serum creatinine level (>133 µmol l⁻¹; OR 0.97; 95 % CI 0.95–0.998; P<0.05) and low peak day 12 anti-TcdA IgG titre (≤3000; odds ratio (OR) 0.07; 95 % CI 0.01–0.99; P<0.05).

Multivariable analysis, controlling for age and underlying disease severity (Charlson index ≥3), was carried out on risk factors that were significantly associated with 30-day mortality. The three independent predictors of mortality were peak WCC concentration (>20×10⁹ cells l⁻¹), high Charlson score (≥3) and low peak day 12 anti-TcdA IgG titre (≤3000; OR 0.97; 95 % CI 0.95–0.998; P<0.05). In a model including high serum creatinine level (>133 µmol l⁻¹), high Charlson score (≥3) and low peak day 12 anti-TcdA IgG titre (≤3000; OR 0.97; 95 % CI 0.95–0.998; P<0.05), significant predictors of mortality were peak WCC concentration (>20×10⁹ cells l⁻¹), high Charlson score (≥3) and low peak day 12 anti-TcdA IgG titre (≤3000; OR 0.97; 95 % CI 0.95–0.998; P<0.05).

Association between bacterial cytotoxin and outcome

Recurrence, 30-day all-cause mortality and attributable mortality on univariate analysis were identified as high serum creatinine level (>133 µmol l⁻¹). Significant risk factors associated with recurrence or 30-day all-cause mortality were high serum creatinine level (>133 µmol l⁻¹) and low peak day 12 anti-TcdA IgG titre (≤3000; OR 0.97; 95 % CI 0.95–0.998; P<0.05). When patients were categorized depending on whether they were infected with a strain of low cytotoxin titre; however, there was no significant association between cytotoxin titre and recurrence or 30-day all-cause mortality overall. All patients for whom death was directly attributable to CDI were infected with a strain of low cytotoxin titre. Of the patients who died by day 30, 13 out of 14 (93 %) were infected with a strain of low cytotoxin titre. Of the patients who had died by 30 days post-diagnosis, there was no significant association between cytotoxin titre and recurrence or 30-day all-cause mortality. When patients were categorized depending on whether they were infected with a strain of low cytotoxin titre, 12 out of 16 patients (75 %) that exhibited a recurrence of CDI were infected with low or high cytotoxin strains, 12 out of 16 patients (75 %) that exhibited a recurrence of CDI were infected with low or high cytotoxin strains, 12 out of 16 patients (75 %) that exhibited a recurrence of CDI were infected with low or high cytotoxin strains. In patients for whom death was directly attributable to CDI, the three independent predictors of mortality were peak WCC concentration (>20×10⁹ cells l⁻¹), high serum creatinine level (>133 µmol l⁻¹) and low peak day 12 anti-TcdA IgG titre (≤3000; OR 0.97; 95 % CI 0.95–0.998; P<0.05).
Table 2. Univariate analysis of host-associated and bacterial-associated risk factors for 30-day all-cause mortality

<table>
<thead>
<tr>
<th>Factor</th>
<th>No. (%) alive at day 30 (n=72)</th>
<th>No. (%) who died by day 30 (n=14)</th>
<th>Mean (±sd) or median values for patients alive at day 30</th>
<th>95 % CI for mean values or range for median values</th>
<th>Mean (±sd) or median values for patients who died by day 30</th>
<th>95 % CI for mean values or range for median values</th>
<th>OR (95 % CI)</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td><strong>Patient factors</strong></td>
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<tr>
<td>Age &gt;70 years</td>
<td>49 (68.06)</td>
<td>11 (78.57)</td>
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<td></td>
<td></td>
<td></td>
<td>1.72 (0.4–6.77)</td>
<td>0.53</td>
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<tr>
<td>Horn’s index &gt;3</td>
<td>6 (8.33)</td>
<td>4 (28.57)</td>
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<td></td>
<td>4.4 (1.05–18.37)</td>
<td>0.03*</td>
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<td>Charlson score</td>
<td></td>
<td>3</td>
<td>0–8 (3)</td>
<td></td>
<td>3</td>
<td>1–9 (2.75)</td>
<td>1.058</td>
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<td><strong>Bacterial factors</strong></td>
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<tr>
<td>High cytotoxin titre (4/5)</td>
<td>10 (13.9)</td>
<td>1 (7.14)</td>
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<td></td>
<td></td>
<td></td>
<td>0.47 (0.06–4.6)</td>
<td>0.68</td>
</tr>
<tr>
<td>Ribotype 027</td>
<td>7 (9.7)</td>
<td>1 (7.14)</td>
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<td></td>
<td></td>
<td></td>
<td>0.71 (0.08–6.31)</td>
<td>1</td>
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<tr>
<td>tcdC deletion</td>
<td>18 (25)</td>
<td>4 (28.57)</td>
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<td></td>
<td></td>
<td>1.2 (0.33–4.3)</td>
<td>1</td>
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<tr>
<td>Binary toxin</td>
<td>23 (31.94)</td>
<td>5 (35.71)</td>
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<td></td>
<td></td>
<td></td>
<td>1.18 (0.35–3.9)</td>
<td>1</td>
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<td><strong>Inflammatory markers</strong></td>
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<tr>
<td>Minimum serum albumin (g dl⁻¹)</td>
<td></td>
<td></td>
<td>2.34 (0.57)</td>
<td>2.21–2.47</td>
<td>2.01 (0.6)</td>
<td>1.7–2.32</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>Peak WCC &gt;20 (×10⁹ l⁻¹)</td>
<td>16 (22.22)</td>
<td>9 (64.29)</td>
<td></td>
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<td></td>
<td></td>
<td>6.3 (1.85–21.47)</td>
<td>0.003*</td>
</tr>
<tr>
<td>Creatinine &gt;133 (μmol l⁻¹)</td>
<td>13 (18.06)</td>
<td>8 (57.14)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6.05 (1.79–20.43)</td>
<td>0.004*</td>
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<td><strong>Immune response</strong></td>
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<tr>
<td>Peak serum anti-TcdA IgG (EU)†</td>
<td></td>
<td></td>
<td>25.2</td>
<td>0–176.4 (62.7)</td>
<td>4.2</td>
<td>0–86 (12.1)</td>
<td>0.01*</td>
<td></td>
</tr>
<tr>
<td>Peak serum anti-TcdB IgG (EU)†</td>
<td></td>
<td></td>
<td>18.0</td>
<td>0–159.3 (75.0)</td>
<td>7.8</td>
<td>0–56.6 (49.2)</td>
<td>0.07</td>
<td></td>
</tr>
</tbody>
</table>

*P<0.05.
†Peak antibody concentration within 12 days of diagnosis.
duration of CDI disease (Warny et al., 1994) and risk of recurrence (Kyne et al., 2001; Warny et al., 1994). Cases of severe CDI are increasing and the role of the host anti-toxin immunoglobulin response in severe CDI and protection from mortality has yet to be defined.

We have shown for the first time that patients exhibiting low serum levels of IgG antibody to TcdA were significantly more likely to die within 30 days of CDI diagnosis. Patients who died within 30 days of CDI diagnosis also exhibited lower anti-TcdB IgG levels. The importance of high serum anti-toxin IgG levels in protecting from mortality due to severe CDI disease has been demonstrated in animals and protection was shown to be mediated by a reduction in systemically disseminated toxins in the serum (Johnson, 2012; Steele et al., 2012). A role for systemic toxaemia in severe human CDI has not yet been established. Future studies are needed to investigate toxaemia in severe CDI using freshly collected sera and highly sensitive toxin assays (He et al., 2009; Steele et al., 2012). This will allow an evaluation of whether the protection associated with high serum anti-toxin IgG levels is due to a reduction in systemic toxaemia (in addition to neutralization of colonic luminal or mucosal toxins). Quantification of toxaemia may also determine whether low anti-TcdA levels are more closely associated with mortality than anti-TcdB IgG levels due to differences in serum concentrations of the respective toxins. It is important to note that the anti-toxin IgG response mounted by CDI patients was independent of age and co-morbidity. The phenomenon of ‘immunosenesence’ suggests that elderly patients are less able to mount an immune response to infection, which was not evident in this study, although the majority of patients studied were elderly, which may bias this finding. In addition, we did not assess the neutralizing ability of anti-TcdA and -TcdB IgG from the patients in this cohort in an in vitro cytotoxicity assay. Although this study was limited by an assessment of IgG quantity but not of functional neutralizing activity, we have shown a significant relationship between IgG levels and protection from mortality.

We also investigated the role of in vitro cytotoxicity of the infecting C. difficile strain in influencing host inflammatory and immune responses. Isolates of ribotype 027 in our study population exhibited a significantly higher in vitro cytotoxin titre than all other ribotypes, which may support claims of the ‘hypervirulent’ status of this strain with respect to cytotoxin production (Kuehne et al., 2010; Thelestam & Chaves-Olarte, 2000). However, some isolates of ribotypes 014 and 001 also exhibited high cytotoxin titres and induced acute inflammatory responses. The TcdC negative regulator has previously been considered responsible for hyper-production of toxins; however, these isolates were negative for mutations/deletions in the tcdC gene and strains of ribotype 078 that contained a 39 bp deletion in tcdC exhibited comparatively low cytotoxin titres. The data presented here therefore support the suggestions of others that the role of TcdC in influencing toxin production is called into question (Bakker et al., 2012; Cartman et al., 2012).

We found that patients infected with strains that exhibited a high cytotoxin titre were significantly more likely to have higher anti-TcdA and -TcdB IgG levels and were more likely to have clinical markers of severe disease, including peak WCC >20 x 10^9 l^-1 and CRP concentrations >230 mg l^-1. This suggests that both the host adaptive and innate immune systems respond sensitively to C. difficile toxin antigens and initiate a response that is reflective of the toxin challenge posed by the infecting strain. This may explain why the majority of patients who were infected with a strain of high cytotoxin titre and exhibited clinical markers of severe disease were still alive 30 days after CDI diagnosis, as these patients were most likely to have a high protective anti-toxin IgG response.

In our study, factors that were significantly associated with an increased risk of mortality after CDI diagnosis were host-associated, including underlying disease severity (Horn’s index), host clinical markers of inflammation and host anti-toxin IgG levels. Of the three non-toxin bacterial-associated factors investigated here (ribotype 027, tcdC deletion and binary toxin), none was significantly associated with mortality. This suggests that laboratory assays that specifically detect the presence of these markers may be of limited use in identifying patients at risk of a fatal outcome of CDI. The outcome of CDI may also be influenced by the host response to other C. difficile antigens, including flagellin, surface-layer proteins and cysteine proteinases; however, these were not investigated in this study.

High cytotoxin titre was not a risk factor for 30-day mortality, and the majority of CDI patients overall (87%) were infected with a strain exhibiting a low cytotoxin titre. This suggests that patients are highly sensitive even to low toxin titres. It is also possible that other non-humoral aspects of the host immune response to C. difficile, including inflammatory cytokine release, vascular leakage and neutrophil migration, may be contributory risk factors for 30-day mortality.

On multivariate analysis, three independent predictors of mortality were identified: high peak WCC, high serum creatinine and low anti-TcdA IgG titre. The data in our study therefore suggest that prediction of the outcome of CDI should be host orientated, by monitoring laboratory markers of inflammation and clinical assessment of patient health. The association between high anti-toxin IgG titre and protection from mortality as shown here provides additional supportive evidence for the development and therapeutic use of active and passive immunotherapies for the management of CDI.

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