Association of Clostridium difficile ribotype 078 with detectable toxin in human stool specimens

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Using a Clostridium difficile glutamate dehydrogenase (GDH) immunoassay and a sensitive C. difficile toxin A/B immunoassay, human stool specimens from patients with diarrhoea (n=1085) were classified as either GDH positive/toxin negative, or GDH positive/toxin positive. Overall, 528/725 (73%) of the GDH-positive/toxin-negative specimens contained viable C. difficile, and 433/528 (82%) of these C. difficile isolates were PCR positive for the toxin gene pathogenicity locus. Overall, 867/1078 (80%) of the GDH-positive specimens contained viable C. difficile, and 433/725 (60%) of the GDH-positive/toxin-negative specimens contained a toxigenic C. difficile strain. The diversity of toxigenic C. difficile ribotypes isolated from toxin-negative specimens (n=433) and toxin-positive specimens (n=339) was significantly different (P<0.0001). Specifically, the presence of ribotype 078 strains was very strongly associated (P<0.0001) with detection of toxin in clinical specimens using a sensitive toxin immunoassay. Specimens positive for ribotype 078 were almost twice as likely to be toxin positive as opposed to toxin negative (risk ratio=1.90, 95% confidence interval 1.64–2.19). In contrast, other circulating ribotypes were seen with similar frequency in specimens with and without detectable toxin. This supports the view that ribotype 078 strains may be more virulent than other common ribotypes in terms of toxin production.

INTRODUCTION

Hospital laboratories commonly diagnose Clostridium difficile infection by detection of toxin A and/or B in stool specimens using enzyme immunoassay (EIA) tests, although these tests have unacceptably low sensitivity when they are used alone. Depending on the toxin EIA used, positive predictive values are as low as 15–56% when testing patient populations with low prevalence (Eastwood et al., 2009). UK guidance now recommends dual testing using two different tests to improve diagnostic accuracy for C. difficile infection (Robotham & Wilcox, 2012). Immunoassays to detect the glutamate dehydrogenase (GDH) antigen are more sensitive than toxin EIA tests for detection of C. difficile, and many clinical laboratories have adopted GDH tests for screening, with secondary or parallel testing using a toxin EIA test. Dual testing using two assays with different sensitivities generates a significant number of discrepant GDH-positive/toxin-negative results, in addition to definitive GDH-positive/toxin-positive results that confirm a diagnosis of C. difficile infection. Because GDH tests are not specific for toxigenic strains of C. difficile, interpreting these discrepant results can be difficult.

The emergence of virulent C. difficile strains is of concern because a highly virulent epidemic C. difficile clone (ribotype 027/NAP1/toxino-type III) has spread globally during the last decade (He et al., 2013). Ribotype 027 strains have been reported to produce significantly more toxin than other strains in vitro. One study reported that 027 strains produced peak titres of toxin A and B that were 16 and 23 times higher, respectively, than other circulating ribotypes (Warny et al., 2005). However, some conflicting data have been published. ‘Robust’ in vitro toxin production by 027 strains was reported in another study, but levels were not significantly different from other strains (Merrigan et al., 2010). A study comparing toxin production by a ribotype 027 strain and a ribotype 001 strain in a human gut chemostat model reported no difference in peak toxin titres, although toxin production by the 027 strain was sustained over a much longer period (Freeman et al., 2007). A comparison of stool toxin levels and disease severity found no association between toxin levels and symptoms, or between in vivo and in vitro toxin production by isolates (Åkerlund et al., 2006). Factors such as the composition of growth media have a dramatic effect on toxin production by C. difficile (Haslam

Abbreviations: CI, confidence interval; d.f., degrees of freedom; EIA, enzyme immunoassay; GDH, glutamate dehydrogenase; PaLoc, C. difficile toxin gene pathogenicity locus; RR, risk ratio.
et al., 1986; Karlsson et al., 1999, 2000; Osgood et al., 1993; Yamakawa et al., 1994) so in vitro studies of toxin production may not be representative of conditions in the gut. In vitro studies often focus on small numbers of strains, and data from large in vivo studies are lacking. The largest study to date comparing definitive laboratory testing with clinical outcomes (Planche et al., 2013) demonstrated that clinical severity and poor outcome were associated with the presence of detectable toxin in stool specimens, using the most sensitive gold standard cell culture cytotoxicity test (CCCT) method, and not simply with presence of a toxigenic strain in the absence of toxin.

Epidemic ribotype 027 strains (Merrigan et al., 2010; Mulvey et al., 2010) and also ribotype 078 strains (Goorhuis et al., 2008) have been described as ‘hypervirulent’ in the literature, although there is no agreed definition of this term. If in vivo toxin production is accepted as a key virulence factor (through higher level or more sustained expression of toxins), we reasoned that genuinely hypervirulent C. difficile strains should be found more often in specimens with detectable toxin than without.

The first aim of this study was to determine whether discrepant GDH-positive/toxin-negative results were due to (i) detection of non-toxigenic C. difficile strains, or (ii) detection of toxigenic strains – either in the absence of toxin, or with toxin levels below the limits of detection using EIA methods. The latter group includes both false-negative toxin test results, and also ‘potential C. difficile excretors’ that may have implications for hospital infection prevention and control (Planche et al., 2013). Our second aim was to test a specific hypothesis about whether different C. difficile ribotypes were associated with the presence of detectable levels of toxin in clinical specimens, by comparing the molecular diversity of C. difficile seen in two groups of clinical specimens (GDH-positive/toxin-negative vs GDH-positive/toxin-positive samples). We were particularly interested in PCR ribotype 078 (NAP7/NAP8/toxinotype V) strains, as the prevalence of this ribotype in Northern Ireland has historically been much higher than in other UK regions, and previous studies have suggested that ribotype 078 strains are highly virulent (Goorhuis et al., 2008) and potentially zoonotic (Jhung et al., 2008).

**METHODS**

Clinical specimens were collected over a 20-month period (August 2012 to March 2014 inclusive) from patients with diarrhea in four Belfast hospitals (Royal Victoria Hospital, Belfast City Hospital, Mater Infirmorium Hospital and Musgrave Park Hospital) and two hospitals in County Down (Lagan Valley Hospital and Downe Hospital), Northern Ireland. Liquid stool specimens were tested on the day of receipt with both a GDH EIA assay (Premier C. difficile GDH; Meridian Bioscience) and a Toxin A/B EIA assay (Premier C. difficile Toxins A&B; Meridian Bioscience) according to the manufacturer’s instructions. Specimens that were positive with either or both of these assays were included in the study, and were processed using a reference spore-culture method (UK Standards for Microbiology Investigations, 2014) to isolate C. difficile. Isolates were further characterized by PCR ribotyping and PCR detection of the C. difficile toxin gene pathogenicity locus (PaLoc).

DNA for PCR testing was extracted by boiling several C. difficile colonies in 300 μl sterile water containing 5% (w/v) Chelex-100 (Sigma-Aldrich) for 10 min, as described previously (Stubbs et al., 1999). Boiled samples were clarified by centrifugation (10 000 g, 1 min). We combined two previously published primer sets into a duplex PCR assay for ribotyping and detection of the C. difficile PaLoc toxin locus using fluorescent fragment analysis. PCR primers for ribotyping (O’Neill et al., 1996; Stubbs et al., 1999) were modified by addition of PET dye to the forward primer (5’-PET-CTGGGTTGA-AGTCTGAAACAGG-3’ and 5’-GGGCCCTTTGTAGCTTGGACC-3’). PCR primers targeting the tcdC gene (Persson et al., 2008) were modified by addition of a 6FAM dye to the forward primer (5’-6FAM-AAAAAGGAGATTGTATTATGTTTCA-3’ and 5’-CAAT-AACTTGATAAACCTACCTCTTCA-3’). Unless specified otherwise, PCR reagents were supplied by Promega. Duplex PCRs (10 μl total) contained the following: GoTaq Flexi buffer, GoTaq DNA polymerase (0.2 U per reaction), oligonucleotide primers (2 pmol per reaction for ribotyping primers; 1 pmol per reaction for tcdC primers; primers were supplied by Applied Biosystems), dNTP mix (80 μM), MgCl2 (3.5 mM), BSA (0.2 mg/ml) and template DNA (2 μl). Thermal cycling conditions were: 94 °C for 3 min, followed by 30 cycles of 94 °C for 45 s, 55 °C for 30 s and 72 °C for 1 min, followed by 72 °C for 5 min. PCR products (2 μl) were diluted by mixing with 20 μl sterile water. Diluted PCR products (1 μl) were added to 9 μl Hi-Di formamide (Applied Biosystems) containing GeneScan 600 LIZ Size Standard (Applied Biosystems) before separation by capillary electrophoresis (POP-7 polymer; 36 cm capillary array) on a 3730xl DNA Analyzer (Applied Biosystems). Fluorescent fragment data were analysed using GeneMarker version 2.4.0 software (SoftGenetics, LLC) and PCR ribotypes were assigned using a library of reference profiles, including all of the most commonly seen ribotypes in Europe. Detection of the tcdC gene confirmed the presence of the PaLoc toxin locus in toxigenic strains.

The 95% confidence intervals (CIs) for the proportion of each ribotype in each group were calculated using binomial distribution (Clopper & Pearson, 1934). Statistical tests (χ² test of independence for proportions and Fisher’s exact test) were done using SPSS version 21 (IBM). The risk ratio (RR) was calculated using Epi Info version 7 (Dean et al., 2011).

Study specimens were tested under approval granted by the Research Ethics Committee, Northern Ireland Health and Social Care (10/NIR01/28).

**RESULTS AND DISCUSSION**

A total of 1085 stool specimens were tested during the study period. Seven (0.6%) of the specimens were GDH negative/toxin positive. Five of these were culture negative and were considered to be false-negative toxin tests. Toxigenic C. difficile strains (ribotype 078 and 193) were isolated from the remaining two specimens, which were considered to be GDH false negative. Excluding these discrepant results, data for the remaining 1078 specimens were as follows.

A total of 528/725 (73%) of the GDH-positive/toxin-negative specimens contained viable C. difficile, and 433/528 (82%) of these C. difficile isolates were PCR positive for the PaLoc toxin gene locus, so were toxigenic. Overall, 867/1078 (80%) of the GDH-positive specimens contained

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**REFERENCES**

Planche, D. J. Fairley and others (1994) to isolate C. difficile. Isolates were further characterized by PCR ribotyping and PCR detection of the C. difficile toxin gene pathogenicity locus (PaLoc).

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viable *C. difficile*, and 433/725 (60%) of the GDH-positive/toxin-negative specimens contained a toxigenic *C. difficile* strain. *C. difficile* was isolated from 339/353 (96%) of the GDH-positive/toxin-positive specimens.

Ribotype data for the *C. difficile* strains isolated are shown in Table 1. Ribotypes with prevalence in both groups of 1.5% or less were combined together in the group labelled ‘Other’. Toxigenic strains in this group included sporadic/non-groupable strains, and the following identified ribotypes: 013, 017, 019, 026, 027, 029, 046, 050, 053, 054, 056, 070, 077, 081, 087, 106, 126, 131, 214, 216 and 258. Non-toxigenic strains (no PaLoc detected by PCR) included non-groupable strains and the following identified ribotypes: 140, 010 and 026. All of the isolates from toxin-positive specimens were PCR positive for the PaLoc toxin locus, and non-toxigenic strains (no PaLoc) were isolated only from GDH-positive/toxin-negative specimens. Only one ribotype 027 strain was isolated during the study, from a toxin-positive specimen.

There was a highly significant difference \( \chi^2 = 68.1 \), degrees of freedom (d.f.) = 11, \( P < 0.0001 \) in the diversity of toxigenic *C. difficile* ribotypes in the toxin-negative group \( (n=433) \), excluding non-toxigenic isolates) and the toxin-positive group \( (n=339) \). Pairwise comparison of 078 with all other ribotypes using Fisher’s exact test gave a highly significant difference \( (P<0.0001) \). A further \( \chi^2 \) test of the entire dataset, excluding ribotype 078, found no significant difference between the specimen groups for all other ribotypes \( (\chi^2 = 16.6, \text{d.f.} = 10, \ P = 0.083) \). This confirmed that the significant difference between the specimen groups could be attributed to the difference in 078 prevalence. We then calculated the RR of a specimen being toxin positive (by EIA) when it is positive for *C. difficile* ribotype 078 \( (\text{RR}=1.90, 95\% \text{ CI } 1.64–2.19) \). A clinical specimen containing ribotype 078 was almost twice as likely to be toxin positive rather than toxin negative by EIA.

Using a relatively insensitive EIA toxin test as part of a highly specific three-stage testing algorithm (GDH, toxin, and culture/PCR testing), we found that ribotype 078 was strongly associated with the presence of detectable toxin in human stool specimens. On this basis, it seems reasonable to conclude that ribotype 078 strains produce more toxin than other circulating ribotypes in Northern Ireland.

Commercial diagnostic tests to detect *C. difficile* toxins are optimized and marketed for qualitative and not quantitative testing. Although toxin EIA assays may be calibrated for quantitative use, this is not routine practice in clinical microbiology laboratories. In this study, we used a qualitative toxin EIA test to classify GDH-positive specimens as either toxin positive or toxin negative, using the absorbance cut-off value specified by the manufacturer. We isolated and characterized *C. difficile* from both specimen groups to determine the prevalence of toxigenic strains in each group. We also investigated the diversity of different *C. difficile* ribotypes in each group using a population biology approach, to test whether specific ribotypes were associated with the presence of detectable levels of toxin. The results were not subject to selection bias, as specimens were referred for testing according to UK national guidance, every *C. difficile* positive specimen during the study period was cultured and all *C. difficile* isolates were characterized.

We isolated toxigenic *C. difficile* strains from 60% of the clinical specimens with a discrepant GDH-positive/toxin-negative specimens contained a toxigenic *C. difficile* strain. *C. difficile* was isolated from 339/353 (96%) of the GDH-positive/toxin-positive specimens.

### Table 1. PCR ribotypes of *C. difficile* isolates from GDH-positive human stool specimens that were either positive or negative by toxin EIA \( (n=867 \text{ isolates}) \)

<table>
<thead>
<tr>
<th>Ribotype</th>
<th>Toxin-positive specimens</th>
<th>Toxin-negative specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of isolates</td>
<td>%*</td>
</tr>
<tr>
<td>078</td>
<td>96</td>
<td>28 (20–37)</td>
</tr>
<tr>
<td>014/020</td>
<td>52</td>
<td>15 (9–23)</td>
</tr>
<tr>
<td>002</td>
<td>28</td>
<td>8 (4–15)</td>
</tr>
<tr>
<td>005</td>
<td>24</td>
<td>7 (3–14)</td>
</tr>
<tr>
<td>015</td>
<td>18</td>
<td>5 (2–11)</td>
</tr>
<tr>
<td>193</td>
<td>15</td>
<td>4 (1–10)</td>
</tr>
<tr>
<td>001</td>
<td>10</td>
<td>3 (1–9)</td>
</tr>
<tr>
<td>018</td>
<td>9</td>
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<tr>
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</tr>
<tr>
<td>023</td>
<td>4</td>
<td>1 (0–6)</td>
</tr>
<tr>
<td>Other</td>
<td>70</td>
<td>21 (14–30)</td>
</tr>
<tr>
<td>No PaLoc</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>339</td>
<td>528</td>
</tr>
</tbody>
</table>

*The 95% binomial CI is shown in parentheses.
Comparing the diversity of *C. difficile* ribotypes in the toxin-negative and toxin-positive specimen groups revealed that ribotype 078 was very strongly associated with the presence of detectable toxin in clinical specimens.

Our approach focused on the use of clinical specimens, rather than characterizing *C. difficile in vitro*, so the data were not affected by changes in toxin expression due to culture conditions or other confounding variables. The toxin EIA used in this study has been reported to be the most sensitive EIA test on the market. It was found in one study to have a specificity of 92%, the highest of nine toxin EIAs evaluated (Eastwood et al., 2009).

A systematic review of nine earlier studies found it had a median specificity of 95%, the highest of five toxin EIAs evaluated (Planche et al., 2008). Nevertheless, if the more sensitive CCCT method had been used, some of the specimens classified as toxin negative using the EIA would have been positive (Planche et al., 2013). The numbers in the GDH-positive/toxin-positive group would increase, with a concomitant decrease in the numbers in the GDH-positive/toxin-negative group. A very large increase in the prevalence of all ribotypes except 078 would be required in the toxin-negative group to affect our conclusion that ribotype 078 is strongly associated with detectable toxin.

Although referred to as hypervirulent (Goorhuis et al., 2008; Mulvey et al., 2010), the evidence that 078 strains cause more severe disease is contradictory. Higher case-fatality rates in cases involving strains with binary toxin genes (i.e. both ribotype 027 and 078) have been reported (Bacci et al., 2011). In contrast, other studies have found no association between the presence of binary toxin genes (in strains including 078) and more severe disease (Goldenberg & French, 2011; Hensgens & Kuiper, 2013), or between ribotype and disease severity, even for ribotype 027 strains (Walk et al., 2012). A case-comparison study comparing a range of patient characteristics for *C. difficile* infection cases with ribotypes 001, 027 and 078 in Northern Ireland has been published (Patterson et al., 2012). These authors found that ribotype 078 was community associated, and mortality for 078 cases was not significantly different from 001 or 027 cases, although the relatively small number of 078 cases (n=29) may have influenced the outcome.

Our data, based on testing of a large and unbiased collection of clinical specimens, indicate that 078 strains are associated with detectable toxin in human stool specimens more often than other circulating ribotypes. This presumably reflects the higher levels of toxin production in vivo, although further study is required to confirm this. In contrast, other circulating ribotypes were seen with similar frequency in specimens with and without detectable toxin. This is the expected outcome if ribotype 078 strains are indeed more virulent than other common ribotypes in terms of toxin production. Further clinical studies are required to determine whether these 078 strains are associated with a more severe disease.

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