Carriage of *Clostridium difficile* in outpatients with irritable bowel syndrome

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Irritable bowel syndrome (IBS) is a common, typically chronic and sometimes disabling gastrointestinal condition of uncertain aetiology. Recently, a variety of links to gastrointestinal infections have been described including the onset of IBS following exposure to enteric pathogens and an apparent predisposition to gastrointestinal infection. The prevalence of *Clostridium difficile* in a population of IBS outpatients (*n*=87) in the absence of established risk factors for the acquisition of *C. difficile* infection was examined. Overall, 5.7% of patients (*n*=5) carried culturable *C. difficile* and 4.6% (*n*=4) of isolates were toxigenic, belonging to toxinotype group 0, compared with 1.1% (*n*=1) for the healthy control group (*n*=88). These isolates were members of toxigenic PCR ribotype groups 005 and 050 (IBS group) and 062 (control group) and were identified further as three individual strains by PFGE. Although no significant difference was observed between IBS patients and healthy volunteers, these findings support the concept that a subpopulation of IBS patients may be susceptible to gastrointestinal infection.

**INTRODUCTION**

Irritable bowel syndrome (IBS) is a common disorder that manifests as abdominal discomfort associated with a variety of disturbances of bowel function (Drossman *et al.*, 2002; Quigley, 2006). Alterations in the intestinal microbiota and the mucosal immune response have been described in IBS and have led to the speculation that this disorder may result from a fundamental abnormality in the interaction between the luminal flora and the host and could be traced to a previous episode of bacterial gastroenteritis (Parkes *et al.*, 2008; Quigley, 2007; Spiller, 2007). Whilst persistent inflammatory IBS may represent a minority of IBS subjects, research on this particular population has shown how, in a susceptible subject, an enteric pathogen that in other circumstances would lead to a self-limited gastroenteritis can result in a persistent inflammatory response and chronic symptoms (Spiller, 2003). Based initially on the assumption that some IBS subjects may harbour small-intestinal bacterial overgrowth, antibiotic usage has been studied in IBS and has demonstrated some benefits in terms of symptom relief (Mättö *et al.*, 2005; Pimentel *et al.*, 2000, 2006; Sharara *et al.*, 2006). These observations raise an important question: is a subgroup of the IBS population susceptible to enteric infection and, specifically, given the current enthusiasm for treating these patients with antibiotics, could some IBS subjects harbour one of the most important opportunistic pathogenic species such as antibiotic-resistant toxigenic *Clostridium difficile*? This pathogen can induce diarrhoeal symptoms similar to those experienced by some in an exacerbation of IBS (Borody *et al.*, 1989). For these reasons, we investigated the carriage of *C. difficile* in IBS patients in the absence of known risk factors to *C. difficile* infection to ascertain whether a predisposition to infection exists.

**METHODS**

**Study populations.** IBS patients (*n*=87, all female) were recruited from gastroenterology clinics at Cork University Hospital, Ireland, and by direct advertisement on the university campus and in the local newspaper. Individuals aged between 19 and 71 years who satisfied the Rome II criteria for the diagnosis of IBS, but for whom organic gastrointestinal diseases such as inflammatory bowel disease were ruled out, were included in the study group. Pregnant individuals, those with known lactose intolerance or immunodeficiency and those who had undergone prior gastrointestinal surgery, with the exception of inguinal hernia repair and appendectomy, were excluded. The

**Abbreviations:** APC, Alimentary Pharmabiotic Centre; CDT, *C. difficile* transferase toxin; IBS, irritable bowel syndrome; PaLoc, pathogenicity locus; VPI, Virginia Polytechnic Institute.
control group comprised 88 healthy female volunteers who were free of gastrointestinal symptoms, with ages ranging from 18 to 65 years.

Isolation and identification of *Clostridium difficile* strains. Faecal samples were collected within 48 h and immediately frozen at −80 °C until required for analysis. *C. difficile* was isolated from ethanol-treated stool samples and selected on cycloserine-cefoxitin egg yolk agar (Lab M). Non-haemolytic and lecithinase-negative colonies of typical morphology were subsequently subcultured onto reinforced clostridial agar (Merck) and assessed for fluorescence under UV light. Isolates were examined by Gram staining and for 1-proline amidopeptidase activity (PRO kit; Remel). *C. difficile* was routinely cultured on fastidious anaerobe agar (Lab M). A single colony was inoculated into 10 ml pre-reduced brain–heart infusion broth (Merck) and grown anaerobically for 18 h at 37 °C. Genomic DNA was extracted as described previously (Cartwright et al., 1995). Presumptive *C. difficile* was identified by 16S rRNA gene sequencing using primer pairs and PCR cycling conditions described previously (Simpson et al., 2003). Amplified fragments of 1.5 kb were purified (QiAquick PCR Purification kit; Qiagen) and sequenced by Lark Technologies. Overlapping contiguous sequences and a consensus sequence were determined using the Seqman program (Lasergene 6; DNASTAR). Sequences homologous to *C. difficile* were identified using BLAST by scanning with all available nucleotide databases for high-scoring sequence pairs.

Toxin production. *In vitro* production of the *C. difficile* TcdA and TcdB toxins was assessed from culture supernatants using enzyme immunoassays. Production of TcdA was demonstrated by a Toxin A assay (Remel), and the Toxin A/B assay (Remel) was used to identify toxin variant isoforms (TcdA−, TcdB+). The following *C. difficile* strains were included for control purposes: ATCC 43600 (TcdA−, TcdB+), ATCC 43593 (TcdA+, TcdB+) and CCUG 20309 (TcdA−, TcdB+).

Antimicrobial susceptibility testing. An Etest system (AB Biodisk) was used to screen isolates for antibiotic resistance to vancomycin, metronidazole, amicillin and clindamycin. The Etest was performed on test cultures inoculated onto fastidious anaerobe agar (Lab M) containing 6% lysed horse blood and interpreted following the manufacturer’s instructions. *Bacteroides fragilis* (ATCC 25285) was included as an internal control for the system. Individual MICs were recorded, and MIC90 was defined as the MIC observed to impede the growth of 90% of the isolates tested (Jamal et al., 2002). Strains were deemed susceptible or resistant to the antibiotic according to documented pharmacological breakpoint values.

Toxinotyping. Toxinotyping is based on a PCR RFLP analysis of variable regions of the tcdA and tcdB genes, encoding the major *C. difficile* toxins TcdA and TcdB. Strains were screened for variation in the first 3.1 kb of tcdB (B1 fragment) and a 3.1 kb fragment spanning the repetitive region of tcdA (A3 fragment) using primer pairs and methodology described previously (Rupnik et al., 1998). The B1 and A3 fragments of the tcdB and tcdA genes were amplified using the BIOTAQ DNA polymerase system (Bioline) and gel purified (QiAquick PCR Purification kit). Fragments were then digested with AclI and HinclI (B1) or EcoRI (A3) (New England Biolabs) and checked for length polymorphisms. Profiles were compared with the reference strains VPI 10463 (toxinotype 0), VIII and VI (reference DNA kindly supplied by Professor M. Rupnik, University of Maribor, Slovenia). The accessory genes tcdC, tcdE and tcdF and genes flanking the pathogenicity locus (PaLoc) integration sites cdw-2 and cdw-3 were amplified using BIOTAQ DNA polymerase using primer pairs and a methodology as described previously (Braun et al., 1996). The tcdC gene was amplified as a 718 bp fragment using primer pairs described previously using the KOD Hot Start Polymerase system (Novagen) and sequenced (Spigaglia & Mastrantonio, 2002). Amino acid sequences were compared with the published tcdC sequence for VPI 10463 (kindly supplied by Dr Scott Curry; Curry et al., 2007), by pairwise and multiple sequence alignments using CLUSTAL W in the MEGALIGN program of Lasergene 6. Finally, internal regions of the two-component binary toxin (actin-specific ADP-ribosyltransferase gene (cdt)) were amplified again with KOD polymerase as described previously (Stubbs et al., 2000); primers cdtApos and cdtArev amplified a 510 bp fragment of the enzymic component (cdtA), and cdtBpos and cdtBrev amplified a 375 bp fragment of the binding component (cdtB). Primers amplifying sequences encompassing the entire gene were used to confirm the absence of binary toxin genes in all cases (Spigaglia & Mastrantonio, 2002); primers BINS and BIN6 amplified a 1158 bp product encompassing the cdtA gene, and BIN7 and BIN8 amplified a 2751 bp product encompassing cdtB. Controls representing *C. difficile* CCUG 20309 and PCR ribotype 001 were used as positive and negative controls for the binary toxin.

PCR ribotyping. PCR ribotyping analysis is based on a comparison of patterns of PCR products generated from the 16–23S rRNA gene intergenic region. Primers complementary to the 3′ end of the 16S rRNA gene and the 5′ end of the 23S rRNA gene were used to amplify the variable length 16–23S intergenic spacer region, as described previously (Stubbs et al., 1999). Ribotype banding patterns were analysed by gel electrophoresis in 3% Metaphor agarose (FMC Bioproducts) for 3.5 h at 65 mA and visualized under UV light. Profiles were compared with clinically significant strains common to Ireland and the UK, including the epidemic strain described as North American pulsed-field type 1, *PCR* ribotype 027 (NAP1/027) and PCR ribotypes 001 and 106. Banding patterns were photographed using a Gel-Doc 2000 System (Bio-Rad) and patterns were subsequently identified by the Anaerobe Reference Laboratory (Cardiff, UK) by comparison with a library of known PCR ribotypes.

PFGE analysis. PFGE was performed as described previously (Clayton et al., 2009). Genomic DNA was prepared in agarose plugs, lysed and digested with SmaI (New England Biolabs). Electrophoresis was performed using the CHEF-DR II system (Bio-Rad) and DNA macrorestriction patterns were stored as TIFF files and imported into BioNumerics software (version 3; Applied Maths) for dendrogramic analysis. Gels were normalized using standard PFGE molecular mass markers (New England Biolabs) ranging from 48.5 to 727.5 kb (ladder) and 2.03 to 194.0 kb (low-range marker). The Dice coefficient of similarity was calculated and UPGMA was used for cluster analysis with optimization set at 1.3% and position tolerance set at 0.9%. The genetic relatedness to NAP1/027 and PCR ribotypes 001 and 106 was also assessed.

Data analysis and statistics. The proportion of patients testing positively for faecal *C. difficile* in IBS patients compared with the proportion in healthy controls was assessed by Fisher’s exact test and values of *P*<0.05 were considered statistically significant.

RESULTS

Carriage of *Clostridium difficile* in IBS outpatients

In this study, 87 female IBS patients whose diagnosis satisfied the Rome II criteria were screened for the presence of *C. difficile*. In terms of IBS subtype, 17 (19.5%) were diarrhoea predominant, 22 (25.3%) were constipation predominant and 48 (55.2%) reported an alternating bowel habit. Table 1 summarizes the frequency of *C. difficile* carriage and the IBS status of individual carriers. Overall, 5.7% (n=5) of patients carried culturable *C. difficile* compared with 1.1% (n=1) for...
the control group. It is noteworthy that only one of the C. difficile-positive patients had diarrhoea-predominant IBS. All isolates were susceptible to metronidazole and vancomycin (MIC₉₀ of 1.5 and 0.75 μg ml⁻¹, respectively) and ampicillin (MIC₉₀ of 1.0 μg ml⁻¹); however, an MIC₉₀ of 4.0 μg ml⁻¹ was found for clindamycin, indicating resistance. Five isolates (four from the IBS group and one from the control group) were toxigenic, as shown by enzyme immunoassays. In this respect, production of the primary toxins TcdA and TcdB was demonstrated using a toxin A/B assay. The toxin A assay indicated that variant strains that produced TcdB alone (TcdA⁻, TcdB⁺) were not isolated.

### Patient isolate ID

<table>
<thead>
<tr>
<th>Patient isolate ID</th>
<th>Patient age (years)</th>
<th>Duration of IBS/type</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC16</td>
<td>24</td>
<td>7 years/IBS-A</td>
</tr>
<tr>
<td>APC17</td>
<td>62</td>
<td>16 years/IBS-D</td>
</tr>
<tr>
<td>APC18</td>
<td>35</td>
<td>9 years/IBS-C</td>
</tr>
<tr>
<td>APC19</td>
<td>52</td>
<td>10 years/IBS-A</td>
</tr>
<tr>
<td>APC21</td>
<td>71</td>
<td>16 years/IBS-A</td>
</tr>
</tbody>
</table>

### Toxinotyping and tcdC analysis

TcdA and TcdB are encoded by the tcdA and tcdB genes and, together with the three accessory genes tcdR, tcdE and tcdC, form part of a 19.6 kb chromosomal unit (PaLoc; Fig. 1) (Braun et al., 1996; Lyerly et al., 1985). Some strains also produce a binary toxin – an actin-specific ADP-ribosyltransferase – referred to as the C. difficile transferase (CDT) toxin (Popoff et al., 1988). CDT is a two-component toxin encoded by the cdtA and cdtB genes, which are located on a specific region of the chromosome outside the PaLoc, termed the C. difficile transferase locus (CdtLoc; Fig. 1) (Carter et al., 2007). The expected PCR fragments for the tcdA and tcdB genes and the accessory genes tcdD (300 bp), tcdE (262 bp) and tcdC (345 bp) of the PaLoc were amplified for all toxigenic strains. Only sequences at the PaLoc integration sites (cdu-2, 162 bp; and cdd-3, 622 bp) were amplified from the non-toxin-producing isolate. Variations may exist in strains that produce both TcdA and TcdB and the toxinotyping scheme describes all strains that exhibit changes within the encoding genes in comparison to a reference strain, VPI 10463 (toxinotype 0). All toxigenic isolates belonged to toxinotype 0, sharing the same PCR-RFLP pattern as that of VPI 10463 following digestion of the B1 (3088 bp) and A3 (3031 bp) fragments. No alterations were observed in the negative regulatory gene (tcdC) following sequencing of the region. As expected of members of this toxinotype group, these strains did not carry binary toxin genes.

### Molecular typing

PCR ribotyping of the six clinical isolates generated four distinct ribotype profiles comprising seven to 11 DNA fragments of ~280–600 bp, namely, PCR ribotype 050 (APC19 and APC18) and PCR ribotype 005 (APC17 and APC16), and one isolate each of PCR ribotypes 060 and 062 (APC21 and APC14, respectively) (Fig. 2). These isolates were further subtyped to the strain level by PFGE. However, PFGE analysis revealed that both isolates within each toxigenic PCR ribotype group were identical strains, i.e. sharing 100% similarity of the Dice coefficient (Table 2).

### DISCUSSION

In this study, we set out to examine whether there was an increased incidence of C. difficile carriage in outpatients suffering from IBS and, moreover, whether IBS was an...
additional risk factor for C. difficile acquisition. To achieve this, we screened a group of IBS patients whose diagnosis satisfied the Rome II criteria and for whom established risk factors for C. difficile acquisition were ruled out.

Of the 87 IBS outpatients tested, five (5.7%) carried culturable C. difficile. Among the control group, one subject (1.1%) carried toxigenic C. difficile (P=0.21, Fisher’s exact test). Despite this fivefold numerical difference in carriage rate between IBS and control subjects, this difference did not achieve statistical significance, probably due to the relatively small size of the populations studied, but this may also reflect the intrinsic heterogeneity of the IBS population. Thus, current symptom-based diagnostic criteria for IBS, such as the Rome II criteria, select for a heterogeneous group of patients whose symptoms may vary considerably in nature, frequency and severity. Indeed, prior studies have related variations in the faecal microbiota in IBS to differences in symptomatology (Kassinen et al., 2007; Malinen et al., 2005). Our findings indicated that a subpopulation of IBS is predisposed to infection with C. difficile. It is also important to note that there was no relationship between IBS subtype and C. difficile carriage, with only one of the five affected patients having diarrhoea-predominant IBS.

What is of particular concern is that four of the strains from the IBS subjects were found to be toxigenic (APC16–19). These strains, together with the toxigenic strain from the control subject, were characterized further in terms of their ability to produce toxins by enzyme immunoassay and by analysis of their PaLoc. In this respect, all toxin-producing isolates belonged to the major toxigenic group 0, sharing the same PCR-RFLP pattern as that of the high-toxin-producing reference strain VPI 10463. Notably, toxinotype 0 is clinically significant and is currently the most prevalent group isolated from symptomatic patients suffering from C. difficile infection (Geric et al., 2004; Rupnik, 2008).

PCR ribotype profile analysis of these five isolates identified four distinct PCR ribotype groups. PCR ribotype group 005 has been associated previously with C. difficile infection and has been included in the top ten most common ribotypes in England and Northern Ireland (John & Brazier, 2005; Wilcox, 2010), whereas 050, 060 and 062 are all rarer groups. Furthermore, the most common epidemic nosocomial ribotypes, 001 and 106 and the hypervirulent 027, isolated previously from inpatients in some Irish and UK hospitals, were not recovered (Barbut et al., 2007; Drudy et al., 2006). Whilst PFGE is a highly discriminatory fingerprinting technique, it did not discriminate the ribotypes further. Both isolates in each PCR ribotype group were 100% identical to each other, revealing the prevalence of two distinct strains in these four IBS patients. These strains are probably reflective of the types circulating in this community setting, and similar analyses and larger numbers of strains are necessary to determine whether they have a particular relevance in IBS. The impact of these strains on the pathogenesis of IBS is unknown, but they could exacerbate existing IBS symptoms due to their toxigenic nature. Additionally, further exposure to risk factors for C. difficile infection and antibiotic therapy, in particular, could precipitate overt and even severe pseudomembranous colitis. At the very least, our findings indicate that IBS patients are not immune from C. difficile carriage, a finding that should be borne in mind prior to recommending broad-spectrum antibiotic use in IBS. Clearly, larger studies in this population are merited.

Table 2. Summary of PCR ribotypes, PFGE pulsotypes and toxigenic status of strains from the IBS and control groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Strain</th>
<th>PCR ribotype</th>
<th>PFGE pulsotype</th>
<th>Toxin production</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBS</td>
<td>APC16</td>
<td>005</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>IBS</td>
<td>APC17</td>
<td>005</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>IBS</td>
<td>APC19</td>
<td>050</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>IBS</td>
<td>APC19</td>
<td>050</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>IBS</td>
<td>APC21</td>
<td>060</td>
<td>3</td>
<td>−</td>
</tr>
<tr>
<td>Control</td>
<td>APC14</td>
<td>062</td>
<td>4</td>
<td>+</td>
</tr>
</tbody>
</table>

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REFERENCES


