INTRODUCTION

*Clostridium difficile* is the main aetiologic agent of antibiotic-associated diarrhoea (CDAD) (Bartlett, 1992; Kelly et al., 1994) and is responsible for both sporadic cases and epidemic outbreaks (Johnson et al., 1990). Most cases are nosocomially acquired, but community-acquired CDAD is being increasingly recognized (Hirschhorn et al., 1994; Kyne et al., 1998).

*C. difficile* can produce two large clostridial toxins (LCTs), toxin A (TcdA), which is mainly enterotoxic, and toxin B (TcdB), which is highly cytotoxic. Both toxins disrupt the cytoskeleton by acting on regulatory proteins involved in actin polymerization (Lyerly et al., 1988). Traditionally, it was considered that toxigenic strains produced both LCTs whereas non-toxigenic strains did not produce either. Some years ago, the isolation of toxigenic strains that produced toxin B only was reported (A+B) (Lyerly et al., 1992) and it was soon demonstrated that this phenomenon was not as rare among clinical isolates as previously thought (Kato et al., 1998; Depitre et al., 1993; Rupnik et al., 2003). CDAD is usually caused by strains producing both LCTs although A+B+ isolates may also cause the disease (Alfa et al., 2000).

The presence of an additional toxin in *C. difficile* has recently been detected. This actin-specific ADP-riboseyltransferase toxin has been designated binary toxin (CDT) due to its two independent proteins, CDTa, the catalytic component, and CDTb, the binding component. *C. difficile* binary toxin is related genetically, immunologically and functionally to the group of clostridial binary toxins, which includes the well known iota toxin of *Clostridium perfringens* (Popoff et al., 1988). It is not clear how the production of the binary toxin by strains of *C. difficile* can determine its virulence. It has been suggested that, although strains that produce CDT only have a relatively low virulence, the toxin could act synergistically in strains that produce both LCTs (Stubbs et al., 2000; Geric et al., 2003). Data regarding the prevalence of CDT in *C. difficile* are scarce, but figures range from 4 to 12.0% (Perelle et al., 1997; Gulke et al., 2001).

The aim of this study was to evaluate the toxigenic status of circulating strains of *Clostridium difficile* in a large Spanish teaching hospital with a high prevalence of CDAD.

METHODS

Strains and identification. Two hundred and twenty isolates of *C. difficile* were collected prospectively over a 6-month period (January–June 2001) from 1154 diarrhoeic stool samples submitted for *C. difficile* investigation (hospitalized patients treated with antibiotics). Samples were cultured on CCFA (cycloserine–cefoxitin–fructose agar) plates which were incubated under anaerobic conditions at 37 °C for 48 h. *C. difficile* isolates were presumptively identified by their colony morphology, yellow colour, ground-glass texture, characteristic horse-dung smell and Gram-stain appearance. Additional biochemical tests were also used (ATB 32A; bioMérieux). Only one isolate was collected from each positive sample.

Toxin detection. The presence of *C. difficile* toxin B was determined by demonstrating a specific cytopathic effect on MRC-5 cells, as described previously (Chang et al., 1979; Bowman & Riley, 1988; Bartlett, 1994), either directly from fecal samples or, if negative, from pure cultures of the micro-organism.

An enzyme immunoassay (CdTOX A OIA; BioStar) was used to detect the presence of toxin A in fecal samples. The test was repeated from pure cultures when a negative result was observed in the direct clinical specimen.

Molecular methods. LCTs and cdt genes were detected by PCR assays. DNA was extracted from pure *C. difficile* cultures using a Chelex resin-based commercial system (InstaGene Matrix; Bio-Rad) following the manufacturer’s recommendations. The tcdA gene was detected using a previously published PCR assay (Kato et al., 1991). Briefly, oligonucleotides 5'-CCC AAT AGA AGA TTC AAT ATT AAG CTT-3' and 5'-GGA AGA AAA GAA CTT CTG GCT CAC TCA GGT-3' were used to prime

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Abbreviation: LCTs, large clostridial toxins.
PCR reactions. Amplifications were performed by 35 cycles at 95 °C for 15 s, 50 °C for 20 s and 72 °C for 40 s. Positive samples produced an amplification product of 251 bp. The tcdB gene was detected using the method of Wolfhagen et al. (1994). Amplification primers were 5'-TAA TAG AAA ACA GTT AGA AA-3' and 5'-TCC AAT CCA AAC AAA ATG TA-3'. Amplification was carried out with 40 cycles at 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min. In positive samples, a fragment of 301 bp was amplified. Binary toxin was also detected by PCR (Stubbs et al., 2000). Two reactions were needed to provide evidence of both components of the toxin. cdaA was detected with primers 5'-TGA ACC TGG AAA AGG TGA TG-3' and 5'-AGG ATT ATT TAG TGG ACC ATT TG-3', whereas cdbB was detected using primers 5'-CTT AAT GCA AGT AAA TAC TGA G-3' and 5'-AAC GGA TCT CTT GCT TCA GTC-3'. In both cases, reactions were subjected to 30 cycles of denaturation at 94 °C for 45 s, annealing at 52 °C for 1 min and elongation at 72 °C for 20 s. Amplification products were detected as bands of 375 and 510 bp for cdaA and cdbB, respectively. All PCR reactions were carried out in a 9700 Applied Biosystems thermocycler. In all cases, amplification products were detected in agarose gels stained with ethidium bromide under UV illumination.

Control strains C. difficile ATCC 9689 (TcdA⁺ TcdB⁻), C. clostridioforme 3268 (TcdA⁻ TcdB⁻), C. difficile 48489 (CDT⁺) and C. difficile 48752 (CDT⁻) were included.

**Bacterial typing.** The genetic diversity of strains sharing the same toxin patterns was analysed by PCR ribotyping, with amplification of the 16S and 23S intergenic regions (Bidet et al., 2000). The primer sequences were 5'-GTT CGG CTG GAT CAC CTC CT-3' (16S primer) and 5'-CCC TGC ACC CTT AAT AAC TTG ACC-3' (23S primer). Amplification conditions consisted of 35 cycles of 1 min for denaturation at 94 °C, 1 min for primer annealing at 57 °C and 1 min for extension at 72 °C. Ribotyping patterns were separated by electrophoresis on MS-8 agarose (3-0 %) at 100 V for 3 h and were analysed with UV light after ethidium bromide staining.

**RESULTS AND DISCUSSION**

The results of LCT detection in the 220 isolates studied are summarized in Table 1. A total of 199 isolates (90-5 %) produced toxin A and in 21 (9-5 %) the toxin was not detected. The PCR method confirmed the presence of the tcdA gene in all 199 isolates and was positive also in 10 of the 21 enzyme immunoassay-negative isolates (95-0 %).

The cytotoxicity assay identified 210 isolates that produced toxin B (95-5 %), and 10 (4-5 %) that did not. All 220 isolates were PCR-positive for the tcdB gene (100 %). Amplification products of tcdA and tcdB genes can be seen in Fig. 1(a) and Fig. 1(b), respectively.

*C. difficile* binary toxin was detected in 10 isolates (4-5 %). In all cases, both components of the CDT were detected (Fig. 2). All 10 CDT-positive isolates were also toxigenic for toxin A and toxin B. Fig. 2 shows examples of positive and negative strains for both components of CDT.

Strains harbouring both LCTs only showed high genetic homogeneity and most of them (170 isolates, 78-0 %) belonged to a single ribotype (R1) (data not shown). The 11 variant strains (TcdA⁻ TcdB⁺) were grouped into five ribotypes (data not shown) and were not associated with any specific unit in the hospital (Table 2). The 10 isolates which were positive for binary toxin showed a high genetic homogeneity belonging to two ribotypes (seven to R8 and

**Table 1.** Number (and percentages) of strains belonging to each toxigenic profile (LCTs) according to both traditional and genetic detection approaches

<table>
<thead>
<tr>
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<th>Traditional methods*</th>
<th>PCR</th>
</tr>
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<tr>
<td>A⁺ B⁺</td>
<td>199 (90-5 %)</td>
<td>209 (95-0 %)</td>
</tr>
<tr>
<td>A⁻ B⁺</td>
<td>11 (5-0 %)</td>
<td>11 (5 %)</td>
</tr>
<tr>
<td>A⁺ B⁻</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A⁻ B⁻</td>
<td>10 (4-5 %)</td>
<td>0</td>
</tr>
</tbody>
</table>

*Enzyme immunoassay for toxin A and cytotoxicity assay on fibroblast cultures for toxin B.

**Fig. 1.** Amplification products of tcdA (a) and tcdB (b) genes. Lanes: 1, 14 and 26, molecular mass marker; 2, reference strain ATCC 9689; 12, negative control (C. clostridioforme 3268); 3–11, 13, 15–25, clinical isolates.
three to R19; data not shown), despite the fact that they
spread throughout different wards and were not clustered
chronologically either (Table 2).

Considering only phenotypic criteria for toxigenicity, 10 of
our 220 isolates (4.5%) would have been considered non-
toxigenic, but they harboured toxin genes. This apparent
disagreement seems to question our present ‘gold standard’
(cytotoxicity assay) for diagnosis. Another consideration is
the clinical significance of detecting genes that could be
unexpressed or even incomplete. It is worth noting that all
our strains were obtained from patients with diarrhoea,
which may explain why we did not find any non-toxigenic
strains.

Binary toxin genes were detected in 4.5% of our strains.
Other authors estimate proportions ranging from 4.0 to
12.0% (Goncalves et al., 2004; Stubbs et al., 2000). All our
strains had both components of the binary toxin, which was
not always the case in the reports of other authors (Perelle et
al., 1997). In our cases, binary toxin was present only in
strains with LCTs, but it has also been reported in strains
without LCTs (Geric et al., 2003).

Data regarding prevalence of different toxins and genes in
C. difficile strains should always be interpreted on the basis
of the clonal distribution in each institution. The genetic
diversity of strains producing both LCTs varies in different
series, depending on the sporadic or epidemic character of
each one (Kato et al., 2001; Spigaglia et al., 2001). Several
authors report a high homogeneity of variant strains (Alfa et
al., 2000; Pituch et al., 2001) and one study reported that
strains producing binary toxin are usually epidemiologically
unrelated and not derived from a common ancestor
(Goncalves et al., 2004), although in this report the isolates
were obtained from 17 different institutions.

Table 2. Chronological, geographical and epidemiological characteristics of the variant
strains (A− B+) and strains showing binary toxin (all A− B+)

<table>
<thead>
<tr>
<th>Reference</th>
<th>Toxin</th>
<th>Ward</th>
<th>Isolation date</th>
<th>Ribotype</th>
</tr>
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<tbody>
<tr>
<td>2526</td>
<td>A− B+</td>
<td>Internal Medicine II</td>
<td>9 January</td>
<td>R21</td>
</tr>
<tr>
<td>30613</td>
<td>A− B+</td>
<td>Nephrology</td>
<td>17 March</td>
<td>R20</td>
</tr>
<tr>
<td>48769</td>
<td>A− B+</td>
<td>Pediatrics</td>
<td>4 May</td>
<td>R2</td>
</tr>
<tr>
<td>56504</td>
<td>A− B+</td>
<td>Pediatric Infectious Diseases</td>
<td>24 May</td>
<td>R2</td>
</tr>
<tr>
<td>56913</td>
<td>A− B+</td>
<td>Traumatology</td>
<td>25 May</td>
<td>R24</td>
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<tr>
<td>58831</td>
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<td>29 May</td>
<td>R21</td>
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</tr>
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</tr>
<tr>
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<td>Gastroenterology</td>
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</tr>
<tr>
<td>70657</td>
<td>A− B+</td>
<td>Urology</td>
<td>27 June</td>
<td>R4</td>
</tr>
<tr>
<td>19181</td>
<td>Binary</td>
<td>Oncology</td>
<td>19 February</td>
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</tr>
<tr>
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<td>Binary</td>
<td>Infectious Diseases</td>
<td>28 March</td>
<td>R8</td>
</tr>
<tr>
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<td>Binary</td>
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<td>R8</td>
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<td>Binary</td>
<td>Internal Medicine III</td>
<td>23 April</td>
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<td>R8</td>
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<td>Binary</td>
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<td>R8</td>
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<tr>
<td>57870</td>
<td>Binary</td>
<td>General Surgery</td>
<td>28 May</td>
<td>R19</td>
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REFERENCES


