Isolates of *Clostridium perfringens* recovered from Costa Rican patients with antibiotic-associated diarrhoea are mostly enterotoxin-negative and susceptible to first-choice antimicrobials

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To assess the prevalence of enterotoxigenic *Clostridium perfringens* among adults suffering from antibiotic-associated diarrhoea in a Costa Rican hospital, faecal samples were analysed from 104 patients by a cultivation approach. The 29 strains obtained, which accounted for an isolation frequency of 28 %, were genotyped and investigated with regard to their *in vitro* susceptibility to penicillin, imipenem, cefotaxime, chloramphenicol and metronidazole using an agar-dilution method. A multiplex PCR for detection of the toxins α , β and ϵ predictably classified all faecal isolates as biotype A. An agglutination assay revealed that only one isolate synthesized detectable amounts of enterotoxin (detection rate 3 %). This result was confirmed by a PCR targeting the *cpe* gene. The spores of the only CPE⁺ isolate did not germinate after incubation for 30 min at temperatures above 80 °C. Most isolates were susceptible to first-choice antimicrobials. However, unusual MICs for penicillin (16 µg ml⁻¹) and metronidazole (512 µg ml⁻¹) were detected in one and three isolates, respectively. The low incidence of enterotoxigenic strains suggests that *C. perfringens* was not a major primary cause of antibiotic-associated diarrhoea in this hospital during the sampling period.

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INTRODUCTION

Diarrhoeic episodes accompanied by loose bowel movements may initiate in 5–25 % of patients receiving prolonged therapy with cephalosporins, clindamycin, broad-spectrum penicillins, trimethoprim and cotrimoxazole (Modi & Wilcox, 2001; Wiström *et al.*, 2001), even 6–8 weeks after treatment discontinuation (Beaugerie & Petit, 2004). This syndrome can be due to direct toxic effects of the above drugs on the intestinal epithelium, the intestinal transit and the digestive function. Nevertheless, it has also been shown to arise due to colonization or overgrowth of anaerobic bacteria such as *Clostridium difficile* (Poutanen & Simor, 2004) and *Clostridium perfringens* (Asha & Wilcox, 2002).

Despite being a minor constituent of the intestinal flora of healthy humans (Fach & Popoff, 1997), a limited number of strains of *C. perfringens* synthesize a pore-forming enterotoxin (*C. perfringens* enterotoxin or CPE) that

induces water and ion leakage in enterocytes and other cell lines (Borriello *et al.*, 1984; Czeuczulin *et al.*, 1993). This effector, which is chromosomally or plasmid-encoded by the *cpe* gene and expressed exclusively during sporulation (Smedley *et al.*, 2004), has been found in strains from all five biotypes known for this species (Petit *et al.*, 1999). However, patients with antibiotic-associated diarrhoea (AAD) have a tendency to carry heat-sensitive *cpe*⁺ isolates of biotype A in their gut (Vela *et al.*, 1999; McClane & Chakrabarti, 2004). This notion is supported by the fact that type B and E strains do not reliably synthesize CPE (Czeuczulin *et al.*, 1996) and also because type E isolates may carry defective *cpe* sequences (Billington *et al.*, 1998).

The present investigation was performed to determine the prevalence of enterotoxigenic strains of *C. perfringens* among 104 patients who developed AAD in the course of their stay in a major Costa Rican hospital. In addition, the resulting strains were genotyped and investigated with respect to their susceptibility to first-choice antimicrobials to gain insight into their diversity. These goals were achieved by means of a cultivation approach, a commercial

Abbreviations: AAD, antibiotic-associated diarrhoea; CPE, *Clostridium perfringens* enterotoxin.

agglutination assay, a PCR assay targeting the *cpe* gene, a multiplex PCR targeting the genes encoding the α , β and ϵ toxins, and an agar-dilution method.

METHODS

Sample collection. This study was performed with samples collected in a major Costa Rican hospital (Hospital San Juan de Dios, San José) between October 2004 and November 2005. Samples of faecal matter were obtained from 104 adult patients who developed diarrhoea following administration of one or a combination of the following antimicrobials: gentamicin, cefotaxime, ceftazidime, clindamycin, cephalixin, rifampicin, ciproxin and oxacillin. All stool samples were collected from symptomatic patients within 3 days of the onset of diarrhoea. Despite exhaustive examination, no prescription pattern could be deduced by analysis of the clinical files. Moreover, there was no trend regarding the reason for which the patients were admitted to the hospital. All stool samples were collected aseptically by hospital personnel, frozen at -80°C and transported to the laboratory on dry ice.

Isolation and identification of *C. perfringens*. The cultivation approach was modified from a protocol originally described by Labbé (2001). Briefly, 1 g faeces was resuspended in 5 ml brain-heart infusion broth (BHI) pre-reduced following the method of Holdeman *et al.* (1977). Following incubation at 44°C for 24 h in a GasPak anaerobic jar, a loopful of cultures exhibiting turbidity and gas production was spread onto oleandomycin/polymixin/sulfadiazine perfringens agar plates. These plates were incubated under anaerobic conditions for 24 h at 44°C . Typical black colonies, as well as atypical white colonies from the same plate, were jointly resuspended in 1 ml pre-reduced BHI and incubated at 44°C for 4 h to confirm the ability of the isolates to quickly grow at this temperature and produce gas. This step was done because some isolates of *C. perfringens* form white colonies on the closely related SPS agar (Morera *et al.*, 1999). Pure cultures on blood agar plates were checked to record the colony morphology, oxygen tolerance and Gram staining of the isolates. In this respect, Gram-positive isolates exhibiting a double zone of haemolysis were tested further with standard procedures (Holdeman *et al.*, 1977) for lecithinase (+) and lipase (–) activity on egg-yolk agar, mobility (–), gelatin hydrolysis (+), indole production (–) and nitrate reduction (variable). Expected results for *C. perfringens* are shown in parentheses.

Detection of CPE. A reverse passive agglutination kit was employed for phenotypic detection of CPE (RPLA; Oxoid). We followed the recommendations of the manufacturer and the US Centers for Disease Control and Prevention, except that cells were heated at 60°C for 10 min rather than at 75°C for 20 min and the tubes were incubated for 72 h under anaerobic conditions instead of for 24 h under aerobic conditions. These modifications aimed to facilitate the growth of injured cells and increase the concentration of CPE in the supernatants, respectively. A Gram stain of the broths confirmed that all isolates sporulated under the described culture conditions. In addition, longer incubation periods gave rise to lower CPE titres. Different colonies from a single plate were pooled, as non-enterotoxigenic and enterotoxigenic strains of *C. perfringens* may co-exist in a host (Asha & Wilcox, 2002). A strong CPE producer isolated in our laboratory from cooked beef meat (isolate LIBA-89) and a reference strain that does not synthesize this toxin (ATCC 13124) were used as controls.

Isolation of genomic DNA. Genomic DNA was isolated from 24 h cultures in BHI broth using a DNeasy Blood & Tissue kit (Qiagen). To ensure complete lysis, cells were incubated for 1 h at 37°C in a

buffer containing 20 mM Tris/HCl (pH 8.0), 2 mM sodium EDTA, 1.2% Triton X-100 and 20 mg lysozyme ml^{-1} . Subsequently, suspensions were treated with proteinase K for 12–14 h at 56°C . Further steps were carried out as recommended by the manufacturer.

PCR-based detection of the *cpe* gene. The ability to amplify the above DNA preparations was checked by PCR using oligonucleotides 8F and 907R, which target conserved sequences in bacterial 16S rRNA genes (Liu *et al.*, 1997). After verifying this requirement, a 426 bp fragment of the *cpe* gene was amplified using recommended amounts (Fach & Popoff, 1997) of the oligonucleotides P145 (5'-GAAAGATCTGTATCTACAACCTGCTGGTCC-3') and P146 (5'-GCTGGCTAAGATTCTATATTTTGTCCAGT-3') and a 2 $\times$ Ready Reaction mix containing *Taq* polymerase (Fermentas). The thermal profile included a denaturation step at 94°C for 5 min and 30 cycles of 30 s at 94°C , 30 s at 55°C and 30 s at 72°C , followed by an extension step of 10 min at 72°C . This assay was validated using genomic DNA from isolate LIBA-89 (*cpe*⁺) and *C. perfringens* ATCC 13124 (*cpe*[–]).

Detection of genes encoding the α , β and ϵ toxins by PCR. The major lethal toxins α , β and ϵ were detected using a multiplex PCR developed by Yoo *et al.* (1997). These reactions contained a 2 $\times$ Ready Reaction mix containing *Taq* polymerase (Fermentas), variable final concentrations of the primer pairs CPA (0.2 μM), CPB (0.4 μM) and CPE (0.4 μM) (Yoo *et al.*, 1997) and 1 μl of the above DNA preparations. The amplification program consisted of 5 min at 94°C , followed by 30 cycles of 1 min at 55°C , 1 min at 72°C and 1 min at 94°C , with an extension step of 10 min at 72°C . The type B strain *C. perfringens* ATCC 3626, which was used as a control, gave rise as expected to three products of 402 bp (α toxin), 236 bp (β toxin) and 541 bp (ϵ toxin). In contrast, the type A strain *C. perfringens* ATCC 13124 only generated a band of 402 bp. Negative controls lacking template DNA were included in all runs. The lack of a reference strain containing the *iap* and *ipb* genes hampered classification of isolates as the E subtype. However, strains from this subtype rarely cause disease in humans (Petit *et al.*, 1999; Smedley *et al.*, 2004). We are also aware of the fact that loss of plasmids harbouring genes for the β and ϵ toxins may result in misclassification of type B and C strains (Petit *et al.*, 1999). For this reason, broths from which DNA was isolated were inoculated with cells maintained in sterile milk at -80°C .

Thermal inactivation of spores from CPE⁺ positive isolates. CPE⁺ isolates were induced to sporulate in pre-reduced modified Duncan medium for 48 h at 35°C . Subsequently, 2 ml of these broths was incubated in separate tubes for exactly 30 min in water baths maintained at 60, 70, 80, 90 and 100°C . To verify the ability of the spores to germinate, 0.1 ml from each heat-treated suspension was inoculated in pre-reduced chopped meat broth and incubated for 48 h at 35°C . The spores of isolate LIBA-89 (CPE⁺) were killed at temperatures above 60°C . In contrast, the spores of strain ATCC 13124 (CPE[–]) did not withstand any of the temperatures tested.

Determination of MICs. The isolates were grown anaerobically for 48–72 h on *Brucella* agar supplemented with 1 mg vitamin K 1^{-1} and 5 mg haemin 1^{-1} . A small amount of cell material from these plates was resuspended in thioglycolate broth and incubated until the turbidity of the broths equalled that of a 0.5 McFarland standard. Subsequently, 3 μl from each culture was placed onto supplemented *Brucella* agar plates modified by the addition of the following at concentrations of 0.5–512 $\mu\text{g ml}^{-1}$: chloramphenicol, metronidazole, penicillin (Sigma-Aldrich), imipenem (Tienam; Merck Sharp & Dohme) or cefotaxime (Dolanex; Rimsa). These drugs were chosen because they are commonly prescribed by Costa Rican physicians for treatment of infections by anaerobic bacteria. MICs were recorded after incubation for 48 h at 37°C . The quality-control strains *Bacteroides fragilis* ATCC 25285^T, *Bacteroides thetaiotaomicron*

ATCC 29741 and *Eggerthella lenta* ATCC 43055 were analysed to validate the procedure. Growth and inoculum controls were performed according to the guidelines of the NCCLS (2004).

RESULTS AND DISCUSSION

We cultivated *C. perfringens* from 29 of the 104 faecal samples analysed (isolation frequency of 28 %). All isolates were classified as biotype A as expected, but only one (CP-35, detection frequency of 3 %) was shown to contain the *cpe* gene and to synthesize detectable amounts of the corresponding enterotoxin. The spores of this isolate lost their viability after incubation for 30 min at temperatures above 80 °C. This may seem unusual given that CPE⁺ isolates associated with AAD are usually heat-sensitive; however, there is a direct relationship between spore heat resistance and sporulation temperature (García-Alvarado *et al.*, 1992) and our isolates were enriched at 44 °C, rather than at 37 °C.

The universal finding of isolates from biotype A was in agreement with their origin, as most *C. perfringens* strains

associated with food poisoning, AAD, sporadic diarrhoea and even some cases of sudden infant death syndrome in humans belong to this subtype (Petit *et al.*, 1999). By contrast, strains from the other biotype linked to humans (biotype C) have almost exclusively been recovered from patients suffering from necrotic enteritis (Petit *et al.*, 1999; Smedley *et al.*, 2004). We cannot exclude the possibility that additional patients carried uncultivable stages or very low numbers of *C. perfringens* in their gut. Nevertheless, the observed isolation frequency (28 %) coincides with previous investigations in equivalent scenarios (Pituch *et al.*, 2002). Although the prevalence of *cpe*⁺ isolates among our collection (3 %) generally matches some values found in the literature (Forward *et al.*, 2003; Asha *et al.*, 2006), higher detection rates have been reported in other countries (Sparks *et al.*, 2001).

The only isolate that could have been implicated in the development of AAD was susceptible to all antimicrobials (CP-35; Table 1). Type A strains of *C. perfringens* are normally susceptible to penicillin, several β -lactams,

Table 1. MICs ($\mu\text{g ml}^{-1}$) of metronidazole, chloramphenicol, imipenem, penicillin and cefotaxime against isolates of *C. perfringens* associated with adult patients suffering nosocomial diarrhoea in a Costa Rican hospital

Isolate	Metronidazole	Chloramphenicol	Imipenem	Penicillin	Cefotaxime
CP-2	2	2	2	1	8
CP-9	2	2	2	1	8
CP-11	2	2	2	1	8
CP-17	2	2	2	1	8
CP-19	2	2	2	1	8
CP-21	2	2	2	1	8
CP-24	4	2	2	1	8
CP-26	2	2	2	1	8
CP-28	≥ 512	2	2	1	8
CP-29	2	4	2	1	8
CP-31	2	2	2	1	8
CP-32	2	2	2	1	8
CP-34	2	2	2	1	8
CP-35*	2	2	2	1	8
CP-39	2	4	2	1	8
CP-40	2	4	2	1	8
CP-42	4	4	2	1	8
CP-44	≥ 512	4	2	1	8
CP-49	2	2	2	1	8
CP-51	2	2	2	1	8
CP-52	2	2	2	1	8
CP-53	2	2	2	1	8
CP-54	≥ 512	8	2	1	8
CP-63	2	4	2	1	8
CP-64	2	2	2	1	8
CP-73	2	2	2	1	8
CP-76	2	2	2	16	8
CP-78	2	4	2	1	8
CP-93	4	4	2	1	8

**cpe*⁺ isolate.

vancomycin, chloramphenicol, clindamycin, cefotaxime, imipenem and metronidazole (Wexler *et al.*, 2001; Goldstein *et al.*, 2003; Finegold *et al.*, 2004; Citron *et al.*, 2005). In agreement with this, all isolates from our collection were sensitive to cefotaxime, chloramphenicol and imipenem (Table 1). A single isolate exhibited phenotypic resistance to penicillin (CP-76, MIC 16 µg ml⁻¹) and another three tolerated up to 512 µg metronidazole ml⁻¹ (CP-28, CP-44 and CP-54). These four isolates, which seemed to be commensal, may function as reservoirs for mobile genes.

Penicillin- and metronidazole-susceptible strains of *C. perfringens* are found frequently (Alexander *et al.*, 1995; Citron *et al.*, 2003). In this species, resistance to penicillin is not conferred by β-lactamase production (Allen *et al.*, 1999) but by decreased binding affinity of the drug to penicillin-binding protein 1 (Murphy *et al.*, 1981; Hecht *et al.*, 1989). In turn, resistance to metronidazole seems to be linked to decreased pyruvate dehydrogenase activity (Sindar *et al.*, 1982) or alterations in flavodoxins or hydrogenases involved in drug activation (Land & Johnson, 1999). To our knowledge, this is the first report of metronidazole-resistant isolates of *C. perfringens* in Costa Rica.

Our data indicated that additional biotic and/or abiotic factors caused the gastrointestinal symptoms developed by these patients. In this context, toxigenic isolates of *C. difficile* were cultivated from almost 30 % of the samples analysed (unpublished data). However, the recovery of isolates with elevated MICs to metronidazole is consistent with the widespread application of this antimicrobial by local physicians. The recovery of enterotoxigenic or non-enterotoxigenic *C. perfringens* from healthy people has not yet been determined in Costa Rica. Research regarding this question and the mechanisms of acquired resistance to metronidazole that circulate in the country will follow.

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