Title: Circulation of highly drug-resistant *Clostridium difficile* ribotypes 027 and 001 in two tertiary-care hospitals in Mexico.

Running title: *Clostridium difficile* drug resistance in Mexico.

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Abstract

Objective: To assess drug susceptibility and characterize *C. difficile* ribotypes in isolates from two tertiary-care hospitals in Mexico.

Methods: Isolates were evaluated for genotyping, antimicrobial susceptibility testing and detection of mutations associated with drug resistance. PCR ribotyping was performed using a combination of gel-based and capillary electrophoresis-based approaches.

Results: MIC_{50} and MIC_{90} were ≥128 mg/L for ciprofloxacin, erythromycin, clindamycin, and rifampicin. There was no reduced susceptibility to metronidazole or tetracycline; however, reduced susceptibility to vancomycin (≥4 mg/L) and fidaxomicin (≥2 mg/L) was detected in 50 (40.3%) and 4 (3.2%) isolates respectively. Furthermore, the *rpo*B Arg505Lys mutation was more frequently detected in isolates with high MIC to rifampicin (≥32 mg/L) (OR = 52.5; 95% CI = 5.17- 532.6; $p < 0.000$).

Of the 124 *C. difficile* isolates recovered; 84 (66.7%) were of ribotype 027, 18 (14.5%) of ribotype 001, and the remainder were other ribotypes (353, 255, 220, 208, 176, 106, 076, 020, 019, 017, 014, 012, 003, and 002).

Conclusion: Ribotypes 027 and 001 were the most frequent *C. difficile* isolates recovered in this study, and demonstrated higher MICs. Furthermore, we found four isolates with reduced susceptibility to fidaxomicin, raising a concern since this drug is currently unavailable in Mexican Hospitals.

Keywords: Drug resistance; Ribotypes; Fidaxomicin; Ribotype 001; *Clostridium difficile.*

Introduction

C. difficile infection (CDI) symptoms may range from mild diarrhea to life-threatening complications. Apart from NAP1/BI/027, other C. *difficile* ribotypes have been associated with severe disease, *e.g.* ribotype 078 affects younger patients and is a frequent causative agent of community-associated disease [1]; ribotype 001 is the dominant strain in eastern Europe and has higher antimicrobial resistance than other ribotypes [2].

First-line treatment for mild to moderate CDI is based on oral administration of metronidazole or vancomycin, with a therapeutic efficacy >70% [3]. In some patients, however, diarrheal symptoms may reappear within days or weeks after having stopped the treatment. Fidaxomicin is a relatively new narrow-spectrum macrocyclic antibiotic drug that is non-inferior to vancomycin in the management of CDI and associated with lower recurrence rates than vancomycin[4]. However, fidaxomicin is currently unavailable in Mexico, thus vancomycin and metronidazole are still the standard treatments for CDI as recommended in the Clinical Practice Guidelines for *C. difficile* Infection in Adults of the Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA). Other therapeutic options that have been proposed in recent years are rifamycins (good *in vitro* activity against *C. difficile*) including rifaximin (for relapsing CDI) [5], and linezolid (protective rather than curative activity) [6].

Resistance to erythromycin may be due to any of more than 20 classes of erythromycin ribosomal methylase (*erm*) genes, including *ermB* [7], which is also related to clindamycin resistance. In *C. difficile,* resistance to fluoroquinolones is usually due to altered DNA gyrase because of nucleotide substitutions in *gyrA* or *gyrB* genes [5]. Resistance to rifamycins or fidaxomicin is mediated by mutations that lead to reduced binding to the β

subunit of RNA polymerase (RpoB) [7,8]. Finally, resistance to linezolid has been related to the presence of the phenicol and lincosamide resistance gene (*cfr*), as described for staphylococci [9].

Although *C. difficile* is an important nosocomial pathogen, little is known about the epidemiology of this microorganism in Mexico. The aims of the present study were to determine the drug susceptibility of Mexican *C. difficile* isolates, particularly to the recently licensed CDI treatment, fidaxomicin, and to study circulating ribotypes in two tertiary-care hospitals in Mexico.

Methods

Settings and study population

We designed an observational study of circulating *C. difficile* ribotypes, drug susceptibility, and drug resistance genes from two hospitals in Mexico: The Hospital Civil of Guadalajara "Fray Antonio Alcalde", is a 1000-bed tertiary-care teaching hospital, in Guadalajara; and the Hospital Universitario "Dr. José Eleuterio González", is a 450-bed tertiary-care teaching hospital in Monterrey.

All patients with confirmed CDI from February 2011 through January 2016 were included in the study. Recurrences were defined as patients with a reappearance of symptoms after resolution of the previous diarrheal episode within 8 weeks or less. As patient information was anonymized and only microbiological data were analyzed,

informed consent was not required. The study was reviewed and approved by the Local Ethics Committee (Approval: 047/16).

Diagnosis of *Clostridium difficile* infection

Clinical diagnosis of CDI was suspected when patients were hospitalized for more than 48 h and had >3 loose stools in the previous 24 h. In the Hospital Civil of Guadalajara, CDI was confirmed by real-time PCR using the Xpert® *C. difficile/Epi* assay (Cepheid, Sunny Vale, CA, USA) and in the Hospital Universitario, the diagnosis was confirmed by the use of the Meridian ImmunoCard ® Toxins A&B (Meridian Bioscience, Inc., Memphis, TN, USA). Some patients were additionally diagnosed by the Xpert® *C. difficile/Epi* assay in Hospital Universitario only at physicians' request.

Culture

Fecal samples from all confirmed CDI cases were treated with absolute ethanol and cultured on *C. difficile* agar (Neogen Corporation, MI, USA) with cefoxitin (16 mg/L) in anaerobic conditions for up to 72 h. Plates were cultured in either an anaerobic jar or anaerobic chamber. Identification was performed by PCR as described [10]. Only one isolate per patient was included in the study.

Antimicrobial susceptibility testing

We tested antimicrobial agents used for CDI treatment such as vancomycin, metronidazole (range from 0.03 mg/L to 128 mg/L), fidaxomicin (range from 0.002 to 8

mg/L), and rifampicin (range from 0.0001 mg/L to 128 mg/L); and also antimicrobials with potential therapeutic use such as linezolid (range from 0.03 mg/L to 128 mg/L). Furthermore, we tested antimicrobials that may be associated with induction of CDI, including ciprofloxacin, moxifloxacin, erythromycin, clindamycin (range from 0.03 mg/L to 128 mg/L), and tetracycline (range from 0.008 mg/L to 128 mg/L).

Susceptibility testing was performed by the agar dilution method using Wilkins-Chalgren agar (Oxoid Limited, Basingstoke, Hampshire, England) and Schaedler's anaerobe broth (Oxoid Limited) [11]. Briefly, overnight cultures in 5 ml of pre-reduced Schaedler's broth were spotted onto plates of Wilkins-Chalgren agar with different concentrations of antibiotics, using a multipoint inoculator (10^4 colony-forming units/spot). An agar plate without an antimicrobial agent was included as a growth control in both aerobic and anaerobic atmosphere and the plates were read after 48 h of incubation at 37°C in an anaerobic environment. *C. difficile* ATCC 700057 was used as quality control.

A stock solution of fidaxomicin (800 mg/L), was prepared in DMSO, then 1 ml of stock was diluted in 5 ml of DMSO and 4 ml of 10% DMSO (final concentrations 80 mg/L); further dilutions were made in 10% DMSO. Stock solutions of remaining antibiotics (2560 mg/L) and dilutions were dissolved accordingly to recommendations of the Clinical and Laboratory Standards Institute (CLSI) document M100-S27.

Mutations associated with drug resistance

To detect mutations associated with resistance to rifampicin or fidaxomicin, two regions of the *rpoB* gene were amplified; for rifampicin, we used previously reported

primers [9], and for fidaxomicin, we designed the primers CdrpoB-FD-F (5'-TCATGGAAAATGGAACACCA-3') and CdrpoB-FD-R (5'-CCAAACCTCCATCTCTCCAA-3'). We designed the primers CdrpoC-VAN-F (5'-GAATGGGTGCTGAAGCTGTA-3) and CdrpoC-VAN-R (5'-GACGGAAACGACCTTGCTTA -3 ') to amplify a region in the *rpoC* gene that has been linked to vancomycin resistance[12]. Furthermore, the presence of the *cfr* gene was investigated by PCR in selected strains as previously described [13].

Sequencing of PCR-purified products was performed by Macrogen Inc. (Seoul, Korea). The sequences were analyzed using the NCBI Basic Local Alignment Search Tool (BLAST).

Typing of isolates

All isolates were typed for *tcdA*, *tcdB, cdtA, cdtB*, and for deletions in *tcdC* by PCR as previously described [14,15]. For ribotyping, amplification of the 16S-23S rRNA intergenic spacer region was conducted by PCR as described [16]. The ATCC strain BAA-1805 (ribotype 027) was used as a control. Selected isolates were ribotyped by capillary electrophoresis at the *C. difficile* Ribotyping Network Reference Laboratory (CDRN) at Leeds Teaching Hospitals Trust, Leeds, UK.

Results

166 **Culture**

167 Samples were cultured in anaerobic jar (n=196, 57.1%) or in anaerobic chamber
168 (n=147, 42.9%). In total, we cultured 343 samples, of which 124 (36.1%) yielded a positive
169 *C. difficile* culture (one isolate per patient). Most of the cases were from the Hospital Civil
170 of Guadalajara (n = 76, 61.3%); the Hospital Universitario accounted for 48 of the cases
171 (38.7%).

172

173 **Antimicrobial susceptibility profiles**

174 Four isolates (3.2%) had reduced susceptibility to fidaxomicin (MIC = 2 mg/L),
175 whereas no isolate was resistant to either tetracycline or metronidazole. MIC_{50} and MIC_{90}
176 were ≥128 mg/L to ciprofloxacin, erythromycin, clindamycin, and rifampicin (Table 1).
177 MIC distributions of CDI treatment drugs (vancomycin, metronidazole and fidaxomicin)
178 are shown in Figure 1.

179

180 **Molecular analysis of drug resistance**

181 When analyzing a region of *rpo*B in fidaxomicin-susceptible isolates (n= 18) and
182 isolates with reduced susceptibility (n=3), we found 7 mutations; one of these caused an
183 amino acid change that was not associated to reduced susceptibility strains (Table 2).
184 Furthermore, the *rpoB* gene was amplified and partially sequenced in 14 rifampicin-
185 susceptible and 22 rifampicin-resistant isolates. We detected 3 mutations that generated
186 amino acid changes in both susceptible and resistant isolates; the Arg505Lys mutation was

187 more frequently detected in resistant isolates (21/22, 95.4%) than in susceptible isolates
188 (4/14, 28.5%) (OR = 52.5; 95% CI = 5.17- 532.6; p<0.000) (Table 2).

189 We also analyzed the *rpoC* gene in 14 vancomycin-susceptible isolates and 12
190 isolates with reduced susceptibility to vancomycin and detected 22 mutations; two of them
191 were associated with an amino acid change (Table 2). The presence of drug resistance
192 genes was analysed in selected isolates (i.e. isolates with the highest and lowest MIC
193 values). The *cfr* gene was not amplified in any of the linezolid-susceptible (n=17) or
194 linezolid-resistant (n=9) *C. difficile* isolates evaluated.

195

196 **Ribotypes**

197 Toxin A and toxin B genes were detected in all isolates. The binary toxin gene was
198 detected in 89 isolates (71.8%), of which 87 (97.8%) contained the *tcdC* 18-bp deletion
199 (Table 3).

200 Eighty-four isolates (67.7%) demonstrated the same ribotype banding patterns to the
201 control strain BAA-1805 (ribotype 027) (Table 2) and we randomly selected twelve strains
202 that were all confirmed to be ribotype 027 by the CDRN (Leeds, UK). Similarly, of 18
203 isolates (14.5%) that demonstrated similar banding pattern to ribotype 001 (90% of
204 similarity), four randomly selected isolates were confirmed to be ribotype 001 by the
205 CDRN (Leeds, UK). The ribotypes and presence of toxin genes of the other isolates are
206 summarized in table 3.

207

Discussion

In our study, ribotype 027 was the predominant strain, accounting for 67.7% of the cases; this ribotype is considered epidemic and has been reported worldwide[17]. In previous publications we have found 027 strain as the predominant ribotype in our settings [18,19], the latter is in contrast to diverse studies were there is a high diversity of ribotypes and 027 stains account for less than 30% [20-22]. The second most frequent ribotype was 001, accounting for 14.5% of the cases; this ribotype is the main ribotype circulating in Korea [23], Czech Republic [2], Croatia [24], and Slovakia [25], however, this is the first report on ribotype 001 circulation in Mexico. This strain has been associated with high drug resistance, including resistance to ciprofloxacin, erythromycin, and clindamycin[24].

Fidaxomicin is an FDA-approved antibiotic for the treatment of CDI[8]. A previous study that included 1,323 isolates showed a MIC_{90} of 0.5 mg/L against *C. difficile*[26]; similarly, Snydman *et al* reported 925 isolates that were inhibited at a fidaxomicin concentration ≤1 mg/L, and the MIC_{90} was 0.5 mg/L [20]. In the present study, we observed a MIC_{90} of 0.06 mg/L; however, we detected 4 ribotype 027 isolates with a fidaxomicin MIC of 2 mg/L; these isolates were recovered from patients with recurrent CDI. This finding is of interest since fidaxomicin is unavailable in Mexican hospitals and none of the patients included in the study were exposed to this drug. However, we performed susceptibility testing only once; nevertheless, the MIC of control strain were reproducible and the four strains with MIC = 2 mg/L were detected in a batch of 36 isolates being tested at the same time. MICs of the remaining 32 isolates were ≤0.125 mg/L.

Reduced susceptibility to fidaxomicin may be due to point mutations in the *rpoB* gene (RNA polymerase subunit β) [12]. We detected one point mutation (Glu1036Gln) in RpoB, but it was not associated with drug resistance (P = 0.489). Leeds *et al.* identified two mutations in *rpoB* that were associated with reduced susceptibility to fidaxomicin; one of them coded a Gln1073Arg substitution and the second was a frameshift after amino acid 117 of a homolog of the MarR family of transcriptional regulators [12]; however, we did not detect these mutations in our strains. Other mutations associated with fidaxomicin reduced susceptibility have been reported, but all of them have been obtained through the serial passage of strains into media containing fidaxomicin [27]. Goldstein *et al.* isolated a strain with a MIC of 16 mg/L to fidaxomicin from a patient with an episode of recurrence during a clinical trial [28]; this isolate harbored a Val1143Gly substitution in *rpoB*. However, the authors did not consider that resistance had developed during the clinical trial and did not explain the clinical relevance of this finding. To our knowledge, there is no report of clinical resistance to fidaxomicin. It is widely known that fidaxomicin reaches high levels in the gut (1,000 μg/g of faeces) [28]; thus, the actual implication of the high MIC in the strains is unknown, considering the lack of reports on clinical resistance and particularly in Mexico, where this drug is not available.

In our study, we detected reduced susceptibility to vancomycin of 40.3% with 48.8% of reduced susceptibility in 027 strains and 33.3% in 001 strains; proportions as high as 87.7% have been reported in ribotype 027 strains [29]. Similarly, to fidaxomicin, it is unlikely that reduced susceptibility impacts on clinical response, due to the high levels of vancomycin reached in the gut (>2000 mg/L); however, among patients from whom we recovered isolates with reduced susceptibility (50 patients), nine died because of CDI and

five of these patients received vancomycin. Nevertheless, other factors may have acted in these patients' response to treatment.

Although this bacterial species has a *vanG* homolog inducible by vancomycin, it does not promote vancomycin resistance [30]. Leeds *et al.* identified an Asp244Tyr substitution in *rpoC* that was associated with reduced susceptibility to vancomycin [12]. Despite our efforts to detect this mutation, it was not found. Leeds *et al.* reported additional mutations: a Pro108Leu substitution in a transferase encoded by murG/CD2725, a stop codon after amino acid 326 in an exonuclease encoded by CD3659, and a single amino acid deletion in an L-serine dehydrogenase (*sdaB*). Therefore, it seems that diverse mechanisms are responsible for reduced susceptibility to vancomycin, particularly those involved in cell wall biosynthesis.

On the other hand, we observed no resistance to metronidazole; similar findings have been reported in other studies [21,31]. Clinical failures with metronidazole treatments have been attributed to the development of heteroresistance and deficiencies in the pharmacokinetics of the drug resulting in low luminal concentrations following oral administration. Resistance to metronidazole is known to be unstable, with the loss of levels of resistance due to laboratory manipulation [5].

High MICs to linezolid has occasionally been described in *C. difficile* [9,32]. Interestingly, the isolates of ribotype 001 showed higher MICs (8-32 mg/L) than 027 isolates (8 mg/L).Although linezolid is not used for the treatment of CDI, linezolid is widely used in the Hospital Civil of Guadalajara for the treatment of nosocomial pneumonia, surgical wound infections, and bloodstream infections not associated with a

catheter. Marín *et al*. found nine isolates resistant to linezolid and were *cfr*-positive *C. difficile* isolates that belonged to the same clonal cluster, suggesting possible horizontal transmission of these strains among patients in their hospital setting [9]. However, we did not detect the *cfr* gene in any of the selected *C. difficile* isolates we studied.

We also found a high proportion of isolates with elevated MICs to rifampicin in 027 strains (95.2%) and 001 strains (83.3%). In contrast, Tenover *et al* found lower proportions (27.5%) of isolates with high MIC to rifampicin [21]. For this antimicrobial agent, a bimodal distribution of MICs has been reported; Norén *et al*. found 80% of strains to with low MIC (>0.016 mg/L) or high MIC (>256 mg/L) [33]. In our isolates, we detected three previously reported amino acid substitutions in RpoB associated with rifampicin resistance: Arg505Lys, His502Asn, and Ile548Met. Curry *et al* [13] reported all three changes, including Arg505Lys, which was present in isolates with MICs >32 mg/L. We also confirmed the importance of this mutation in rifampicin-resistant isolates (OR = 52.5, CI 5.17- 532.6, p = 0.000). Similarly, the authors reported an Ile548Met change in isolates with MICs >32 mg/L, however, in the *C. difficile* strains evaluated in the present study, this change was not associated with rifampicin resistance (p = 0.074).

Our study has some limitations. First, we were unable to recover all isolates from all samples, in fact, the recovery rate was low. The low recuperation can be attributed to the medium used, which does not incorporate sodium taurocholate as spore germinant; and the use of ethanol to eliminate any vegetative organisms that survived freezing. Consequently, the isolates are not homogeneously distributed throughout the study period, making it difficult to study distribution over time, and perhaps generating bias on ribotype prevalence; second, clinical diagnosis was not confirmed in a uniform way. Apart from

297 differences in diagnosis, this may have contributed to the low recovery of isolates; and
298 finally, data of ribotyping in 027 and 001 strains were mainly extrapolated from primary
299 results of conventional electrophoresis.

300 In conclusion, this is the first report on drug susceptibility of *C. difficile* ribotypes
301 circulating in Mexico. Ribotypes 027 and 001 were the most frequent and highly drug
302 resistant; furthermore, we found four isolates with reduced susceptibility to fidaxomicin,
303 raising a concern since this drug is unavailable in Mexican Hospitals. The clinical relevance
304 of these findings needs to be addressed to fully understand the epidemiology of CDI in
305 Mexican hospitals.

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309

310 **Author Disclosure Statement**

311 No competing financial interests exist.

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