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

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

4

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34 **Abstract**

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

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

41 **Results:** MIC₅₀ and MIC₉₀ were ≥ 128 mg/L for ciprofloxacin, erythromycin,
42 clindamycin, and rifampicin. There was no reduced susceptibility to metronidazole or
43 tetracycline; however, reduced susceptibility to vancomycin (≥ 4 mg/L) and fidaxomicin
44 (≥ 2 mg/L) was detected in 50 (40.3%) and 4 (3.2%) isolates respectively. Furthermore,
45 the *rpoB* Arg505Lys mutation was more frequently detected in isolates with high MIC to
46 rifampicin (≥ 32 mg/L) (OR = 52.5; 95% CI = 5.17- 532.6; $p < 0.000$).

47 Of the 124 *C. difficile* isolates recovered; 84 (66.7%) were of ribotype 027, 18
48 (14.5%) of ribotype 001, and the remainder were other ribotypes (353, 255, 220, 208, 176,
49 106, 076, 020, 019, 017, 014, 012, 003, and 002).

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

54
55 **Keywords:** Drug resistance; Ribotypes; Fidaxomicin; Ribotype 001; *Clostridium difficile*.

56

57 **Introduction**

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

63 First-line treatment for mild to moderate CDI is based on oral administration of
64 metronidazole or vancomycin, with a therapeutic efficacy >70% ³. In some patients,
65 however, diarrheal symptoms may reappear within days or weeks after having stopped the
66 treatment. Fidaxomicin is a relatively new narrow-spectrum macrocyclic antibiotic drug
67 that is non-inferior to vancomycin in the management of CDI and associated with lower
68 recurrence rates than vancomycin⁴. However, fidaxomicin is currently unavailable in
69 Mexico, thus vancomycin and metronidazole are still the standard treatments for CDI as
70 recommended in the Clinical Practice Guidelines for *C. difficile* Infection in Adults of the
71 Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases
72 Society of America (IDSA). Other therapeutic options that have been proposed in recent
73 years are rifamycins (good *in vitro* activity against *C. difficile*) including rifaximin (for
74 relapsing CDI) ⁵, and linezolid (protective rather than curative activity) ⁶.

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

80 subunit of RNA polymerase (RpoB) ^{7,8}. Finally, resistance to linezolid has been related to
81 the presence of the phenicol and lincosamide resistance gene (*cfrr*), as described for
82 staphylococci ⁹.

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

88

89 **Methods**

90

91 **Settings and study population**

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

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

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

103

104 **Diagnosis of *Clostridium difficile* infection**

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

112

113 **Culture**

114 Fecal samples from all confirmed CDI cases were treated with absolute ethanol and
115 cultured on *C. difficile* agar (Neogen Corporation, MI, USA) with cefoxitin (16 mg/L) in
116 anaerobic conditions for up to 72 h. **Plates were cultured in either an anaerobic jar or**
117 anaerobic chamber. Identification was performed by PCR as described¹⁰. Only one isolate
118 per patient was included in the study.

119

120 **Antimicrobial susceptibility testing**

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

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

128 Susceptibility testing was performed by the agar dilution method using Wilkins-
129 Chalgren agar (Oxoid Limited, Basingstoke, Hampshire, England) and Schaedler's
130 anaerobe broth (Oxoid Limited) ¹¹. Briefly, overnight cultures in 5 ml of pre-reduced
131 Schaedler's broth were spotted onto plates of Wilkins-Chalgren agar with different
132 concentrations of antibiotics, using a multipoint inoculator (10⁴ colony-forming units/spot).
133 An agar plate without an antimicrobial agent was included as a growth control in both
134 aerobic and anaerobic atmosphere and the plates were read after 48 h of incubation at 37°C
135 in an anaerobic environment. *C. difficile* ATCC 700057 was used as quality control.

136 A stock solution of fidaxomicin (800 mg/L), was prepared in DMSO, then 1 ml of
137 stock was diluted in 5 ml of DMSO and 4 ml of 10% DMSO (final concentrations 80
138 mg/L); further dilutions were made in 10% DMSO. Stock solutions of remaining antibiotics
139 (2560 mg/L) and dilutions were dissolved accordingly to recommendations of the Clinical
140 and Laboratory Standards Institute (CLSI) document M100-S27.

141

142 **Mutations associated with drug resistance**

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

145 primers⁹, and for fidaxomicin, we designed the primers CdrpoB-FD-F (5'-
146 TCATGGAAAATGGAACACCA-3') and CdrpoB-FD-R (5'-
147 CCAAACCTCCATCTCTCCAA-3'). We designed the primers CdrpoC-VAN-F (5'-
148 GAATGGGTGCTGAAGCTGTA-3) and CdrpoC-VAN-R (5'-
149 GACGGAAACGACCTTGCTTA -3 ') to amplify a region in the *rpoC* gene that has been
150 linked to vancomycin resistance¹². Furthermore, the presence of the *cfr* gene was
151 investigated by PCR in selected strains as previously described¹³.

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

155

156 **Typing of isolates**

157 All isolates were typed for *tcdA*, *tcdB*, *cdtA*, *cdtB*, and for deletions in *tcdC* by PCR
158 as previously described^{14,15}. For ribotyping, amplification of the 16S-23S rRNA intergenic
159 spacer region was conducted by PCR as described¹⁶. The ATCC strain BAA-1805
160 (ribotype 027) was used as a control. Selected isolates were ribotyped by capillary
161 electrophoresis at the *C. difficile* Ribotyping Network Reference Laboratory (CDRN) at
162 Leeds Teaching Hospitals Trust, Leeds, UK.

163

164 **Results**

165

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. MIC₅₀ and MIC₉₀
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 *rpoB* 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 *cfp* 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

208 **Discussion**

209 In our study, ribotype 027 was the predominant strain, accounting for 67.7% of the
210 cases; this ribotype is considered epidemic and has been reported worldwide¹⁷. In previous
211 publications we have found 027 strain as the predominant ribotype in our settings^{18,19}, the
212 latter is in contrast to diverse studies where there is a high diversity of ribotypes and 027
213 stains account for less than 30%²⁰⁻²². The second most frequent ribotype was 001,
214 accounting for 14.5% of the cases; this ribotype is the main ribotype circulating in Korea²³,
215 Czech Republic², Croatia²⁴, and Slovakia²⁵, however, this is the first report on ribotype
216 001 circulation in Mexico. This strain has been associated with high drug resistance,
217 including resistance to ciprofloxacin, erythromycin, and clindamycin²⁴.

218 Fidaxomicin is an FDA-approved antibiotic for the treatment of CDI⁸. A previous
219 study that included 1,323 isolates showed a MIC₉₀ of 0.5 mg/L against *C. difficile*²⁶;
220 similarly, Snyderman *et al* reported 925 isolates that were inhibited at a fidaxomicin
221 concentration ≤ 1 mg/L, and the MIC₉₀ was 0.5 mg/L²⁰. In the present study, we observed a
222 MIC₉₀ of 0.06 mg/L; however, we detected 4 ribotype 027 isolates with a fidaxomicin MIC
223 of 2 mg/L; these isolates were recovered from patients with recurrent CDI. This finding is
224 of interest since fidaxomicin is unavailable in Mexican hospitals and none of the patients
225 included in the study were exposed to this drug. However, we performed susceptibility
226 testing only once; nevertheless, the MIC of control strain were reproducible and the four
227 strains with MIC = 2 mg/L were detected in a batch of 36 isolates being tested at the same
228 time. MICs of the remaining 32 isolates were ≤ 0.125 mg/L.

229 Reduced susceptibility to fidaxomicin may be due to point mutations in the *rpoB*
230 gene (RNA polymerase subunit β)¹². We detected one point mutation (Glu1036Gln) in
231 RpoB, but it was not associated with drug resistance ($P = 0.489$). Leeds *et al.* identified two
232 mutations in *rpoB* that were associated with reduced susceptibility to fidaxomicin; one of
233 them coded a Gln1073Arg substitution and the second was a frameshift after amino acid
234 117 of a homolog of the MarR family of transcriptional regulators¹²; however, we did not
235 detect these mutations in our strains. Other mutations associated with fidaxomicin reduced
236 susceptibility have been reported, but all of them have been obtained through the serial
237 passage of strains into media containing fidaxomicin²⁷. Goldstein *et al.* isolated a strain
238 with a MIC of 16 mg/L to fidaxomicin from a patient with an episode of recurrence during
239 a clinical trial²⁸; this isolate harbored a Val1143Gly substitution in *rpoB*. However, the
240 authors did not consider that resistance had developed during the clinical trial and did not
241 explain the clinical relevance of this finding. To our knowledge, there is no report of
242 clinical resistance to fidaxomicin. It is widely known that fidaxomicin reaches high levels
243 in the gut (1,000 $\mu\text{g/g}$ of faeces)²⁸; thus, the actual implication of the high MIC in the
244 strains is unknown, considering the lack of reports on clinical resistance and particularly in
245 Mexico, where this drug is not available.

246 In our study, we detected reduced susceptibility to vancomycin of 40.3% with
247 48.8% of reduced susceptibility in 027 strains and 33.3% in 001 strains; proportions as high
248 as 87.7% have been reported in ribotype 027 strains²⁹. Similarly, to fidaxomicin, it is
249 unlikely that reduced susceptibility impacts on clinical response, due to the high levels of
250 vancomycin reached in the gut (>2000 mg/L); however, among patients from whom we
251 recovered isolates with reduced susceptibility (50 patients), nine died because of CDI and

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

254 Although this bacterial species has a *vanG* homolog inducible by vancomycin, it
255 does not promote vancomycin resistance³⁰. Leeds *et al.* identified an Asp244Tyr
256 substitution in *rpoC* that was associated with reduced susceptibility to vancomycin¹².
257 Despite our efforts to detect this mutation, it was not found. Leeds *et al.* reported additional
258 mutations: a Pro108Leu substitution in a transferase encoded by *murG/CD2725*, a stop
259 codon after amino acid 326 in an exonuclease encoded by *CD3659*, and a single amino acid
260 deletion in an L-serine dehydrogenase (*sdaB*). Therefore, it seems that diverse mechanisms
261 are responsible for reduced susceptibility to vancomycin, particularly those involved in cell
262 wall biosynthesis.

263 On the other hand, we observed no resistance to metronidazole; similar findings
264 have been reported in other studies^{21,31}. Clinical failures with metronidazole treatments
265 have been attributed to the development of heteroresistance and deficiencies in the
266 pharmacokinetics of the drug resulting in low luminal concentrations following oral
267 administration. Resistance to metronidazole is known to be unstable, with the loss of levels
268 of resistance due to laboratory manipulation⁵.

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

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

278 We also found a high proportion of isolates with elevated MICs to rifampicin in 027
279 strains (95.2%) and 001 strains (83.3%). In contrast, Tenover *et al* found lower proportions
280 (27.5%) of isolates with high MIC to rifampicin ²¹. For this antimicrobial agent, a bimodal
281 distribution of MICs has been reported; Norén *et al.* found 80% of strains to with low MIC
282 (>0.016 mg/L) or high MIC (>256 mg/L) ³³. In our isolates, we detected three previously
283 reported amino acid substitutions in RpoB associated with rifampicin resistance:
284 Arg505Lys, His502Asn, and Ile548Met. Curry *et al* ¹³ reported all three changes, including
285 Arg505Lys, which was present in isolates with MICs >32 mg/L. We also confirmed the
286 importance of this mutation in rifampicin-resistant isolates (OR = 52.5, CI 5.17- 532.6, *p* =
287 0.000). Similarly, the authors reported an Ile548Met change in isolates with MICs >32
288 mg/L, however, in the *C. difficile* strains evaluated in the present study, this change was not
289 associated with rifampicin resistance (*p* = 0.074).

290 Our study has some limitations. First, we were unable to recover all isolates from all
291 samples, in fact, the recovery rate was low. The low recuperation can be attributed to the
292 medium used, which does not incorporate sodium taurocholate as spore germinant; and the
293 use of ethanol to eliminate any vegetative organisms that survived freezing. Consequently,
294 the isolates are not homogeneously distributed throughout the study period, making it
295 difficult to study distribution over time, and perhaps generating bias on ribotype
296 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.

306

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309

310 **Author Disclosure Statement**

311 No competing financial interests exist.

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