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1 **Virulence of new variant strains of *Clostridium difficile* producing only toxin A or binary**  
2 **toxin in the hamster model**

3

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## **ABSTRACT**

Pathogenesis of *Clostridium difficile* has been linked to production of toxins, the large toxins A and B and also the binary toxin CDT. Until recently toxin A was only found in combination in clinical strains with the toxin B unlike toxin B or CDT which were found alone in toxigenic variants. New toxigenic variants of *C. difficile* detected in our laboratory from patients suffering of diarrhea or severe colitis, including a variant producing only the toxin A, were tested for virulence in the hamster model which displays the clinical features of *C. difficile* disease. Hamsters infected with a strain producing only the toxin B induced similar clinical signs, time to death from infection and histological damages, as compared to a hypervirulent strain 027. No mortality or clinical signs of infection but cecal histological damages were found with the variant producing only the toxin A. The *C. difficile* variant strain producing only CDT was able to kill one hamster out of 7; nevertheless the surviving animals presented a few little alteration of the cecum.

## 34 INTRODUCTION

35 *Clostridium difficile* is a Gram positive anaerobic and spore-forming bacterium responsible of  
36 nosocomial digestive infections in most developed countries. The symptoms of *C. difficile*  
37 infections (CDI) range from mild self-limited diarrhea to life-threatening pseudomembranous  
38 colitis [1].

39 The major virulence factors of *C. difficile* are two large protein toxins, that share the same  
40 structural features and the same mechanism of action, the glycosylation of the Rho and Ras  
41 proteins [2,3]. This leads to the disruption of the epithelial cell cytoskeleton and tight junctions  
42 between cells and the loss of epithelial integrity and more extensively tissue damage mainly in  
43 the colon. Despite their similarities in their sizes and modular structures, those two toxins do  
44 not recognize the same receptor for their binding (see review from Aktories [2]).

45  
46 Genes *tcdA* and *tcdB* coding for TcdA and TcdB, respectively, are part of a region of 19.6-kb  
47 named PaLoc for Pathogenicity Locus. The PaLoc also harbors 3 other genes, *tcdR* and *tcdC*,  
48 involved in the positive and negative transcriptional regulation of the toxin genes, respectively,  
49 and *tcdE*, which encodes a putative holin required for the efficient secretion of TcdA and TcdB  
50 [4,5]. PaLoc is found mostly in the same genomic location and is replaced in non-toxicogenic  
51 strains by a highly conserved 75/115 bp non-coding region [6]. Further the genetic  
52 polymorphism found in the PaLoc can be explored by a PCR-restriction method called  
53 toxinotyping [7].

54 Most *C. difficile* clinical strains usually produce both toxins, but some strains only produce  
55 TcdB (strains A<sup>-</sup>B<sup>+</sup>). These strains belong to different toxinotypes and the most frequent ones,  
56 (toxinotype VIII), are characterized by the presence within the PaLoc of a modified *tcdA* gene  
57 leading to a truncated non-active toxin A. They have been described worldwide and have  
58 caused large outbreaks across several Asian countries [8]. In 2015, Janezic et al. described a

59 new toxinotype strain (toxinotype XXXII) characterized by a lack of whole genes *tcdA* and  
60 *tcdC* and an atypical integration site of the PaLoc [9]. More recently, a new variant strain A<sup>+</sup>B<sup>-</sup>  
61 strain, harboring *tcdA* but not *tcdB* [10], was isolated for the first time in humans and showed  
62 also different site of integration.

63 The emergence of genetic variants of *tcdA* and *tcdB* genes raised the question of the role of  
64 TcdA and TcdB in *C. difficile* pathogenesis. The contribution of the two toxins to the disease  
65 has been evaluated through diverse *in vitro* or *in vivo* models. One of them, the hamster model,  
66 emerged because it mirrors many clinical aspects of human CDI, with hemorrhagic caecitis,  
67 and eventually death. Using this model and different mouse infection models, Lyras et al.  
68 [11,12] analyzed isogenic toxin mutants from two different *C. difficile* strains including  
69 JIR8094 a derivative of *strain 630* strain 630 and demonstrated that the A<sup>+</sup>B<sup>-</sup> mutant was less  
70 virulent than both the parental A<sup>+</sup>B<sup>+</sup> and the A<sup>-</sup>B<sup>+</sup> mutant strains, whereas in studies performed  
71 by Kuehne et al. [13,14] in the hamster model, the A<sup>+</sup>B<sup>-</sup> mutant from strains 630Δ*erm* or  
72 R20291 were almost as virulent as the parental and the A<sup>-</sup>B<sup>+</sup> mutant strains. Despite this  
73 discrepancy, which has been putatively related to SNPs occurring between the two genetically  
74 close parental strains, these results suggest strongly that TcdB plays a major role in the  
75 occurrence of clinical signs.

76 A third toxin called binary toxin (CDT) is also produced by 23% of the toxigenic strains  
77 including the hypervirulent clones 027 and 078 [15]. Briefly, the binary toxin of *C. difficile*  
78 targets the small G protein and its actin-specific ADP-ribosylating activity result in the  
79 disorganization of cytoskeleton of the cells (see review from Gerding [16]). Although its  
80 precise role in the pathogenesis remains unclear, it is recognized that it could act synergistically  
81 with the TcdA and TcdB toxins. In particular, it was shown that CDT enhances the virulence  
82 of *C. difficile* in mice via the suppression of a protective host eosinophilic response [17]. It

83 has also been shown that CDT induces microtubule protrusions at the cell surface, which wrap  
84 and embed bacterial cells, thereby largely increasing the adherence of *C. difficile* [18].

85 Role of the different toxins has already been evaluated by testing isogenic mutants of *C.*  
86 *difficile* in the hamster model. But to our knowledge, the virulence of clinical strains producing  
87 only the toxin A or the binary toxin has never been tested in this model. Therefore the objective  
88 of this study was to test the ability of two clinical variant strains, putatively involved in human  
89 infections, to reproduce the symptomatology in hamsters.

90

## 91 RESULTS

### 92 Typing and antimicrobial susceptibility of the *C. difficile* strains

93 Following MLST determination, the five *C. difficile* strains were classified in five different  
94 sequence types (STs), ST 11 (clade 5), ST 1 (clade 2) and ST15 (clade 1) for CD13-073 (A<sup>-</sup>B<sup>-</sup>  
95 CDT<sup>+</sup>), CD13-125 (A<sup>+</sup>B<sup>+</sup>CDT<sup>+</sup>) and CD15-159 (NT) respectively, and two new ST for RA09-  
96 070 (A<sup>+</sup>B<sup>-</sup>CDT<sup>-</sup>) and CD10-165 (A<sup>-</sup>B<sup>+</sup>CDT<sup>+</sup>) (Table 1).

97 The alleles profile of the new ST of the strain RA09-070 (A<sup>+</sup>B<sup>-</sup>CDT<sup>-</sup>) was: *adk* = 15, *atpA* = 21,  
98 *dxr* = 31, *glyA* = 38, *recA* = 21, *sodA* = 36 and *tpi* = 30. The alleles profile of the new ST of  
99 CD10-165 (A<sup>-</sup>B<sup>+</sup>CDT<sup>+</sup>) was: *adk* = 13, *atpA* = 18, *dxr* = 22, *glyA* = 33, *recA* = 18, *sodA* = 31  
100 and *tpi* = 26.

101 *C. difficile* CD10-165 (A<sup>-</sup>B<sup>+</sup>CDT<sup>+</sup>) and RA09-070 (A<sup>+</sup>B<sup>-</sup>CDT<sup>-</sup>) were susceptible to all  
102 antibiotics tested (erythromycin, clindamycin, moxifloxacin, metronidazole, vancomycin). *C.*  
103 *difficile* CD13-073 (A<sup>-</sup>B<sup>-</sup>CDT<sup>+</sup>) was susceptible to the same antibiotics except moxifloxacin  
104 (MIC=12 mg/L). *C. difficile* CD13-125 (A<sup>+</sup>B<sup>+</sup>CDT<sup>+</sup>) and CD15-159 (NT) were susceptible to  
105 metronidazole and vancomycin but highly resistant to erythromycin (MIC>256 mg/L). *C.*  
106 *difficile* CD15-159 (NT) was also resistant to clindamycin (MIC>256 mg/L) and CD13-125  
107 (A<sup>+</sup>B<sup>+</sup>CDT<sup>+</sup>) was resistant to moxifloxacin (MIC>32 mg/L).

108

### 109 The colonization rates of the hamsters is similar for the five *C. difficile* strains

110 Clindamycin-pretreated hamsters were orogastrically challenged with spores of each *C. difficile*  
111 strain. We evaluated the degree of colonization by the five *C. difficile* strains by numeration of  
112 both vegetative forms and spores in fecal pellets. At day 2 post-infection, the level of  
113 colonization, evaluated by the fecal clearance of the bacteria (in CFU/g feces), was not  
114 significantly different for the five strains: 6.1 10<sup>6</sup> for RA09-070 (A<sup>+</sup>B<sup>-</sup>CDT<sup>-</sup>) to 1.5 10<sup>7</sup> for  
115 CD13-073 (A<sup>-</sup>B<sup>-</sup>CDT<sup>+</sup>). In hamsters infected with RA09-070 (A<sup>+</sup>B<sup>-</sup>CDT<sup>-</sup>), CD13-073 (A<sup>-</sup>B<sup>-</sup>

116 CDT<sup>+</sup>) and CD15-159 (NT), the *C. difficile* colonization level remained stable, above 10<sup>6</sup>  
117 CFU/g feces at day 14 when the hamsters were euthanized (Fig. 1).

118

### 119 **Clinical monitoring of hamsters**

120 All hamsters given CD13-125 (A<sup>+</sup>B<sup>+</sup>CDT<sup>+</sup>) or CD10-165 (A<sup>-</sup>B<sup>+</sup>CDT<sup>+</sup>) died within 96 h, mostly  
121 between 48 h and 72 h, and were subject to wet tail, diarrhea, and loss of weight. In contrast, all  
122 hamsters given RA09-070 (A<sup>+</sup>B<sup>-</sup>CDT<sup>-</sup>) or CD15-159 (NT) were still alive at day 14 (Fig. 2).  
123 No differences were found in term of clinical observations between the non-toxigenic strain  
124 and the RA09-070 (A<sup>+</sup>B<sup>-</sup>CDT<sup>-</sup>) strain; in particular, an increase of about 10% of weight was  
125 observed at the end of the experiment for the hamsters infected with these 2 strains (see Table  
126 S1). But hamsters infected with strain CD13-073 (A<sup>-</sup>B<sup>-</sup>CDT<sup>+</sup>) presented two phenotypes.  
127 Whereas 6 out of 7 hamsters presented no clinical signs and survived until the end of the assay,  
128 one died at day 4 without clinical signs prior to death.

129

### 130 **Histological changes**

131 In order to assess the impact on cecal histology of the five *C. difficile* strains, sections of cecal  
132 tissues were sampled at the time of death for all hamsters. Strains CD10-165 (A<sup>-</sup>B<sup>+</sup>CDT<sup>+</sup>) and  
133 CD13-125 (A<sup>+</sup>B<sup>+</sup>CDT<sup>+</sup>) produced very similar major histological changes and representative  
134 histopathology in the cecum is shown in Fig. 3. There was an acute haemorrhagic congestion  
135 resulting in edema within the submucosa and the lamina propria, a neutrophil infiltration, an  
136 acute hyperplasia and finally we found an extensive epithelial damage with disruption of the  
137 villi (mean histological score= 10.63 and 10.29 for strains CD10-165 (A<sup>-</sup>B<sup>+</sup>CDT<sup>+</sup>) and CD13-  
138 125 (A<sup>+</sup>B<sup>+</sup>CDT<sup>+</sup>) respectively in Fig. 4).

139 As expected, hamsters infected with the non toxigenic strain presented an intact cecum (Fig. 3  
140 and mean histological score of 0.14 in Fig. 4). In contrast, despite lack of mortality and

141 characteristic clinical signs of CDI, neutrophilia infiltration was observed with an extensive  
142 hyperplasia for hamsters infected with RA09-070 (A<sup>+</sup>B<sup>-</sup>CDT<sup>-</sup>) (Fig. 3). Epithelial cell loss was  
143 sometimes present but unclotted red blood cells were rarely found. The mean histological score  
144 of 4.5 was significantly different (p<0.0001) compared to the score of the TcdB producers  
145 CD10-165 (A<sup>-</sup>B<sup>+</sup>CDT<sup>+</sup>) and CD13-125 (A<sup>+</sup>B<sup>+</sup>CDT<sup>+</sup>). Interestingly, the 6 of the 7 hamsters  
146 infected with the strain CD13-073 (A<sup>-</sup>B<sup>-</sup>CDT<sup>+</sup>) that survived to the challenge presented little  
147 alteration of the cecum with essentially some neutrophilia infiltration and the mean histological  
148 score was significantly higher compared to the score of the non toxigenic strain (mean  
149 histological score= 1.1 and 0.3 respectively, p<0.05). The seventh (that died within 48h post-  
150 infection) presented an important inflammation and an acute hyperplasia with an individual  
151 histological score of 9 (Fig. 3 and 4).

152

## 153 Discussion

154 *C. difficile* can produce three different toxins and it is established that human CDI is a  
155 multi-factor occurrence which depends of the *C. difficile* strain and host susceptibility. The  
156 purpose of this study was to assess the pathogenesis of new variant strains isolated from  
157 symptomatic patients in the hamster model [11,13]. Our panel of strains included a strain  
158 producing only the binary toxin CDT, a strain producing only TcdA and, as controls for the  
159 validation of the model, a non-toxigenic strain, a hypervirulent strain which produced the three  
160 toxins TcdA, TcdB and CDT and a strain producing TcdB and CDT.

161

162 The validation of our model was assessed because clinical strains TcdB producing  
163 strains CD13-125 (A<sup>+</sup>B<sup>+</sup>CDT<sup>+</sup>) and CD10-165 (A<sup>-</sup>B<sup>+</sup>CDT<sup>+</sup>) induced similar clinical signs in  
164 hamsters, time to death from infection and severe histological damages, highlighting the fact

165 that TcdB appears clearly sufficient to induce CDI in the hamster model. The pathogenicity of  
166 the two variants producing only one toxin, TcdA or CDT was however different.

167 Therefore RA09-070 (A<sup>+</sup>B<sup>-</sup>CDT<sup>-</sup>) which was recently characterized as the first clinical  
168 strain producing TcdA without TcdB [10] was isolated from a patient with an antibiotic-  
169 associated diarrhea who recovered after metronidazole treatment, strongly suggesting that *C.*  
170 *difficile* was implicated in the disease. The genome of the strain possesses no copy of the  
171 classical PaLoc but a gene coding *tcdA* within a PaLoc encompassing *tcdR* and a new putative  
172 CDS coding a complete domain related to the prophage BhlA/UviB located far from the usual  
173 integration site of the PaLoc in *C. difficile*. Also this strain does not harbor genes coding the  
174 binary toxin CDT.

175 Regarding its virulence in the hamster model, no mortality was found as well as absence of  
176 clinical signs, notably the absence of diarrhea, with this strain that caused diarrhea in a patient.  
177 Challenge of hamsters with this strain showed however a kinetic of colonization identical to the  
178 four other strains used in this study indicating that its *in vivo* fitness was not implicated in the  
179 lack of the virulence of the strain. Those observations are consistent with previous study of  
180 Lyras et al. [11] in which a *tcdB* toxin gene mutant of a strain JIR8094, a derivative of *C.*  
181 *difficile* strain 630, was not associated with mortality in the hamster model. However the same  
182 authors [12] found lethality with a significant delay for an isogenic mutant A<sup>+</sup>B<sup>-</sup> of a Canadian  
183 epidemic strain (strain M7404). Other genetic factors could modulate the virulence of the  
184 strain, highlighting that the criteria of lethality in the hamster model for strain producing only  
185 TcdA should be used carefully.

186 RA09-070 (A<sup>+</sup>B<sup>-</sup>CDT<sup>-</sup>) infection was associated to cecal histological damages but at a lower  
187 extent in comparison with the TcdB producing strains (whether or not strains produced TcdA or  
188 CDT), with significant neutrophil infiltration and hyperplasia, signs of inflammation. Batah et  
189 al. [19] demonstrated in a mouse model that the two toxins of *C. difficile* strains 630 or R20291

190 act in synergy with the flagellin, the major component of the filament of the flagella of the  
191 bacteria to produce the strong pro-inflammatory response observed during the pathogenesis of  
192 *C. difficile*. As RA09-70 (A<sup>+</sup>B<sup>-</sup>CDT<sup>-</sup>) possesses the different regulons coding the proteins of the  
193 flagella (Genbank accession Number JPPA00000000), we suggest that TcdA and the flagellin  
194 of this strain are responsible of the inflammation observed in our model. The fact that a strain  
195 producing only TcdA is involved in a human CDI must be taken into account in term of  
196 diagnostic strategy. This new variant also illustrates the genetic heterogeneity of *C. difficile* and  
197 further studies must be done to explain its pathogenicity in humans in contrast to what is  
198 observed in hamsters.

199  
200 Strain CD13-073 (A<sup>-</sup>B<sup>-</sup>CDT<sup>+</sup>) is a *C. difficile* strain producing only CDT. The A<sup>-</sup>B<sup>-</sup>  
201 CDT<sup>+</sup> strains are classified mostly as toxinotype XI and are seldom isolated in human infection  
202 [20] [21]. However their prevalence in animals, as observed in diarrheic calves, is more  
203 important [22]. The strain was isolated from a 56-years old patient treated with imipenem and  
204 gentamicin for pneumonia. He developed a pseudomembranous colitis and after metronidazole  
205 treatment the clinical signs resolved. Using the hamster model, Geric et al found no mortality  
206 or symptoms with three different strains producing CDT only [23]. In contrast CD13-073 (A<sup>-</sup>B<sup>-</sup>  
207 CDT<sup>+</sup>) infection resulted in death for one animal out of seven with histological damages similar  
208 to those found with TcdB-producing strains, and was able to produce mild histological damages  
209 in the remaining surviving animals. Death of 3 hamsters out of 8 was also observed by Kuehne  
210 et al. [14] with a mutant strain producing CDT only, and animals presented atypical symptoms  
211 of CDI with moderate clinical signs as wet tail. Variation of virulence of strains producing only  
212 CDT in the hamster model suggest that individual susceptibilities and/or presence of bacterial  
213 factors may modulate the effect of binary toxin and may further explain why these variants of  
214 *C. difficile* are rarely isolated in humans.

215

216 Overall, we found that a severe inflammation in the ceca of infected hamsters is  
217 correlated with mortality of the animals. Also our results confirmed, using clinical strains and  
218 not laboratory mutants, the major role of TcdB and a less important role of TcdA for *C. difficile*  
219 virulence in the hamster model. Several factors could explain this observation: degree of  
220 dysbiosis of the host, presence in the microbiota of other species of bacteria promoting the  
221 effect of the pathogen, role of the innate immunity.... This highlights the limitation of the  
222 animal model.

223

224

225

## 226 **MATERIALS AND METHODS**

### 227 ***C. difficile* strains and spore preparation**

228 Four toxigenic *C. difficile* clinical isolates CD13-125 (A<sup>+</sup>B<sup>+</sup>CDT<sup>+</sup>), CD10-165 (A<sup>-</sup>B<sup>+</sup>CDT<sup>+</sup>),  
229 RA09-070 (A<sup>+</sup>B<sup>-</sup>CDT<sup>-</sup>) and CD13-073 (A<sup>-</sup>B<sup>-</sup>CDT<sup>+</sup>) were used in this study. One non-toxigenic  
230 *C. difficile* clinical isolate CD15-159 (A<sup>-</sup>B<sup>-</sup>CDT<sup>-</sup> or NT), isolated from a liver abscess in 2015,  
231 was added as a negative control (Table 1). Strain CD13-125 (A<sup>+</sup>B<sup>+</sup>CDT<sup>+</sup>) belongs to the  
232 epidemic PCR-ribotype 027 and was isolated from severe CDI cases in Marseille in 2013.  
233 Strain CD13-073 (A<sup>-</sup>B<sup>-</sup>CDT<sup>+</sup>) belongs to the PCR-ribotype 288 and was isolated from a patient  
234 with pseudomembranous colitis, in Bordeaux in 2012. Strains RA09-070 (A<sup>+</sup>B<sup>-</sup>CDT<sup>-</sup>) and  
235 CD10-165 (A<sup>-</sup>B<sup>+</sup>CDT<sup>+</sup>), isolated from patients suffering from AAD and PMC, respectively,  
236 were characterized by whole genome sequencing as reported elsewhere [10]. .

237 The identification of the strains was confirmed using Matrix-assisted laser desorption  
238 ionization (Maldi) time-of-flight (Tof) mass spectrometry (Brucker) and the glutamate  
239 dehydrogenase (GDH) component of the C. diff Quik Chek Complete® assay (Abbot).  
240 Spores were prepared using the method described by Siani et al. [24]. Briefly, strains were  
241 plated on solid media Wilkins-Chalgren agar for 48 h at 37°C in an anaerobic chamber. Then,  
242 plates were incubated aerobically at room temperature for 5 days. The entire bacterial lawn was  
243 scraped off using a sterile swab and resuspended in 1 ml sterile water. To kill the vegetative  
244 cells, bacterial suspensions were mixed with ethanol (vol/vol) and incubated for 30 min at room  
245 temperature. Then, the suspension was centrifuged (3000 r/min., 20 min.) and the pellet was  
246 resuspended in 1 ml ethanol, sonicated 1 min, and washed in cold water three times. Spore  
247 stocks were stored at +4°C and were enumerated by plating on Wilkins-Chalgren agar  
248 containing taurocholate 0,1%.

249

## 250 **Typing**

251 MLST was performed according to the scheme developed by Griffith et al. [25]. Briefly, seven  
252 housekeeping genes (*adk*, *atpA*, *dxr*, *glyA*, *recA*, *sodA* and *tpi*) were amplified by PCR and  
253 sequenced as previously described. The sequence type (ST) was determined as a combination  
254 of alleles identified by comparing sequences with sequences available in the *C. difficile* MLST  
255 database (<http://pubmlst.org/cdifficile/>). Toxinotyping was performed according to the scheme  
256 developed by Rupnik et al. [26].

257

## 258 **Determination of Minimum inhibitory concentration (MIC)**

259 Susceptibility testing for erythromycin, clindamycin, moxifloxacin, metronidazole and  
260 vancomycin was carried out using the E-test (bioMérieux) on Brucella Blood Agar  
261 (bioMérieux) as described elsewhere [27].

262

263 **Hamster infection model**

264 Adult *Mesocricetus auratus* female hamsters (weight, 80-100 g) were obtained from Charles  
265 River Laboratories and were housed individually in polypropylene isolator cages fitted with  
266 filter covers holding disposable polyester air filters. All food, water, bedding, cages, wire lids  
267 and filter covers were autoclaved before being used and food and water were given *ad libitum*.

268 Animals were randomly distributed in five groups (minimum of seven animals per group) and  
269 five days before infection, each animal was given orogastrically a single dose of clindamycin at  
270 50 mg/kg as described previously [28]. On day 0, each animal received  $10^4$  spores of the  
271 adequate strain and animals were monitored daily for signs of disease such as weight loss, lack  
272 of activity, wet tail and diarrhea until death or during 14 days for surviving hamsters which  
273 were then euthanized. Bacterial colonization was monitored at 2-, 4-, 7-, 10- and 14-days post  
274 challenge for surviving hamsters. Briefly, a suspension at 10 mg/ml of stools was homogenized  
275 in PBS and then was diluted from  $10^{-1}$  to  $10^{-4}$ ; 100  $\mu$ l of each dilution was inoculated on  
276 ChromID agar (bioMérieux) in duplicate and plates were incubated in anaerobic atmosphere at  
277 37°C for 48 hours before counting of the colonies.

278 All animal experiments were conducted according to the European Union guidelines for the  
279 handling of laboratory animals  
280 ([http://ec.europa.eu/environment/chemicals/lab\\_animals/home\\_en.htm](http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm)) and were approved by  
281 the Central Animal Care Facilities and Use Committee of University Paris-Sud (agreement C-  
282 92-019-01; protocol number 2012-108).

283

284 **Histological staining and scoring**

285 Cecal sections were collected from hamsters and were fixed in 10% formalin for a minimum of  
286 2 h then transferred in ethanol 70% for 18 h and finally paraffin embedded. Sections of 3  $\mu$ m

287 were stained with hematoxylin and eosin and analyzed in a blind manner using the  
288 histopathological scoring scheme from Buckley et al. [29]. Briefly, extent and depth of  
289 inflammation were evaluated with 4 different criteria on a scale ranging from 0 to 3: neutrophil  
290 infiltration (0, no neutrophil accumulation; 1, local acute neutrophilic infiltrate; 2, extensive  
291 submucosal neutrophil accumulation; 3, transmural neutrophilic infiltrate), haemorrhagic  
292 congestion (0, normal tissue; 1, engorged mucosal capillaries; 2, submucosal congestion with  
293 unclotted blood; 3, transmural congestion with unclotted blood), hyperplasia (0, no epithelial  
294 hyperplasia; 1, two-fold increase in thickness; 2, threefold increase in thickness; 3, fourfold  
295 increase in thickness) and percent of epithelial barrier involvements (0, no damage; 1, less than  
296 10% mucosal barrier involved; 2, between 10% and 50% of mucosal barrier involved; 3, more  
297 than 50% mucosal barrier involved).

298

### 299 **Statistical analysis**

300 All analysis were performed using Graph Pad Prism 5. Results of colonization kinetics were  
301 expressed as mean number of total CFU per g of fecal pellets. Results of histology were  
302 expressed as mean (+/- standard deviation) histology score per strain.

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307

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**TABLE 1. Clinical and biological data on the 5 strains.**

Strain	Ribotype		Clinical symptoms /treatment	Origin Year of isolation	Outcome	Phenotype	Genotype (PaLoc)
	ST	Age					
CD10-165	New	74 y	PMC	Villefranche sur Saône	Death attributable to CDI the day	A <sup>-</sup> B <sup>+</sup> CDT <sup>+</sup>	<i>tcdR</i> <sup>+</sup>
	New		None	(France), 2010	after CDI diagnosis		<i>tcdB</i> <sup>+</sup>
	Clade C-I						<i>tcdE</i> <sup>+</sup>
							<i>tcdA</i> <sup>-</sup>
						<i>tcdC</i>	
RA09-070	New	60 y	AAD	Cambrai (France)	Resolution of diarrhea	A <sup>+</sup> B <sup>-</sup> CDT <sup>-</sup>	<i>tcdR</i> <sup>+</sup>
	New		Oral MTZ 500 mg x3	2009	No relapse		<i>tcdB</i> <sup>-</sup>
	Clade 5		10 days				<i>tcdE</i> <sup>-</sup>
							<i>tcdA</i> <sup>+</sup>
						<i>tcdC</i>	
CD13-125	RT 027	73 y	AAD	Marseille (France)	unknown	A <sup>+</sup> B <sup>+</sup> CDT <sup>+</sup>	<i>tcdR</i> <sup>+</sup>
	ST 1		unknown	2013			<i>tcdB</i> <sup>+</sup>
	Clade 2						<i>tcdE</i> <sup>+</sup>
							<i>tcdA</i> <sup>+</sup>
						<i>tcdC</i> <sup>+</sup> -18pb deletion in	

								<i>tcdC</i>
CD15-159	010	54 y	Liver abscess	Créteil (France)	Cure	AB <sup>-</sup> CDT <sup>-</sup>	PaLoc replaced by 115	
	15		MTZ 500 mgx3 IV	2015			bp	
	Clade 1							
CD13-073	288	56y	PMC	Bordeaux (France)	Resolution of diarrhea	AB <sup>-</sup> CDT <sup>+</sup>	<i>tcdR</i> <sup>-</sup>	
	ST 11		Oral MTZ 250 mgx4	2012	No relapse		<i>tcdB</i> <sup>-</sup>	
	Clade 5						<i>tcdE</i> <sup>-</sup>	
							<i>tcdA</i> <sup>-</sup>	
							<i>tcdC</i> <sup>+</sup> , -39 bp deletion in <i>tcdC</i>	
PMC,	pseudomembranous	colitis;	MTZ,	metronidazole;	AAD,	antibiotic-associated	diarrhea	

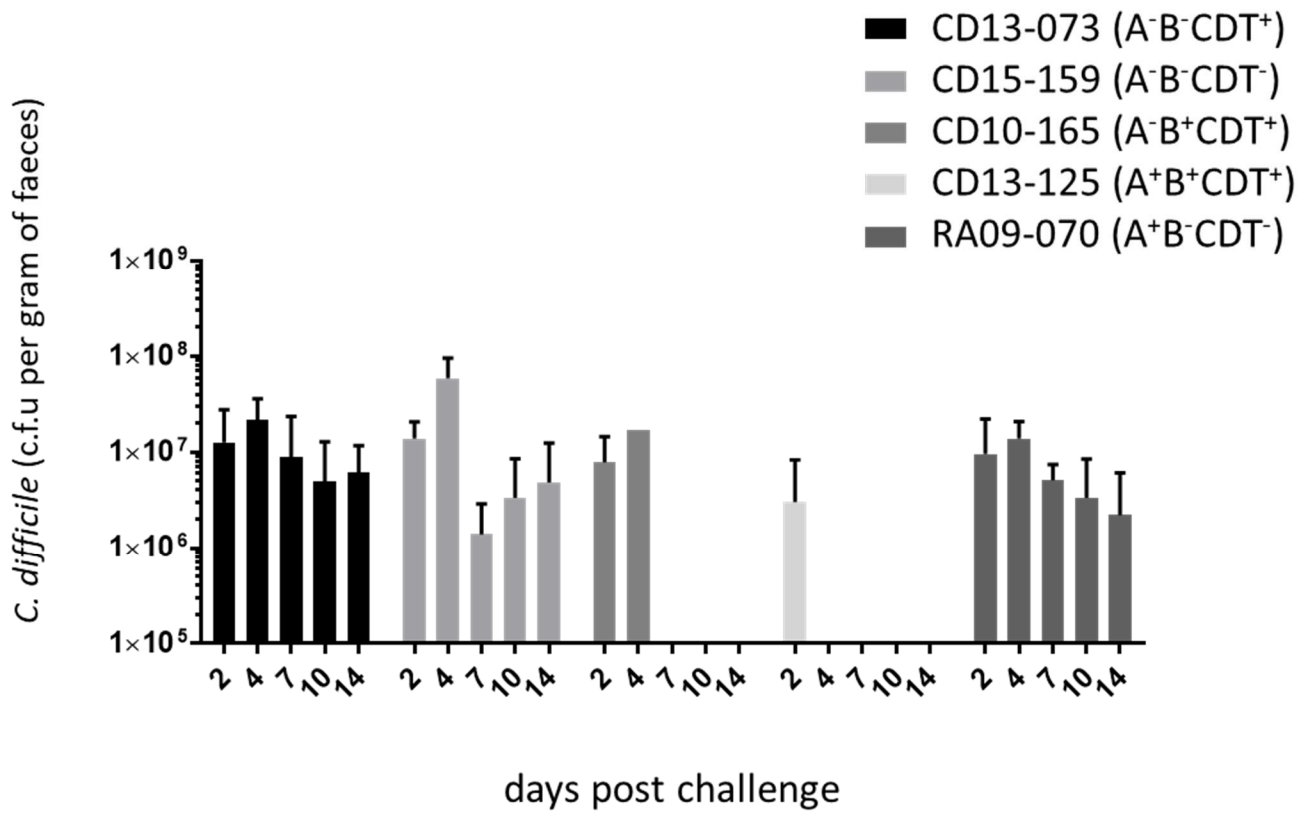
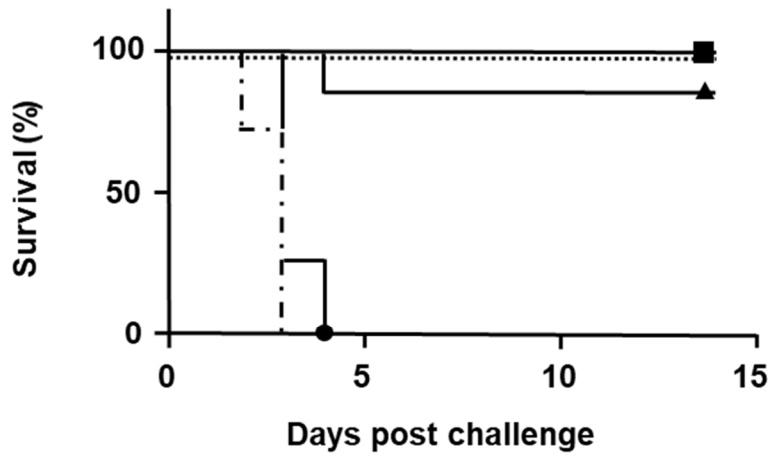


Fig. 1. Colonization kinetics of *C. difficile* strains in hamsters. Colonization of each *C. difficile* strain was monitored in faecal samples by numerating both vegetative and spore forms at day 2, 4, 7, 10 and 14 for the surviving hamsters.



- ▲— CD13-073 (A<sup>-</sup>B<sup>-</sup>CDT<sup>+</sup>) n=7
- ..... CD15-159 (NT) n=7
- RA09-070 (A<sup>+</sup>B<sup>-</sup>CDT<sup>-</sup>) n=8
- · - CD13-125 (A<sup>+</sup>B<sup>+</sup>CDT<sup>+</sup>) n=7
- CD10-165 (A<sup>-</sup>B<sup>+</sup>CDT<sup>+</sup>) n=8

Fig. 2. Kaplan Meyer survival plots for hamster infected with the five different strains after a single dose of clindamycin (n=7 or 8 per group).

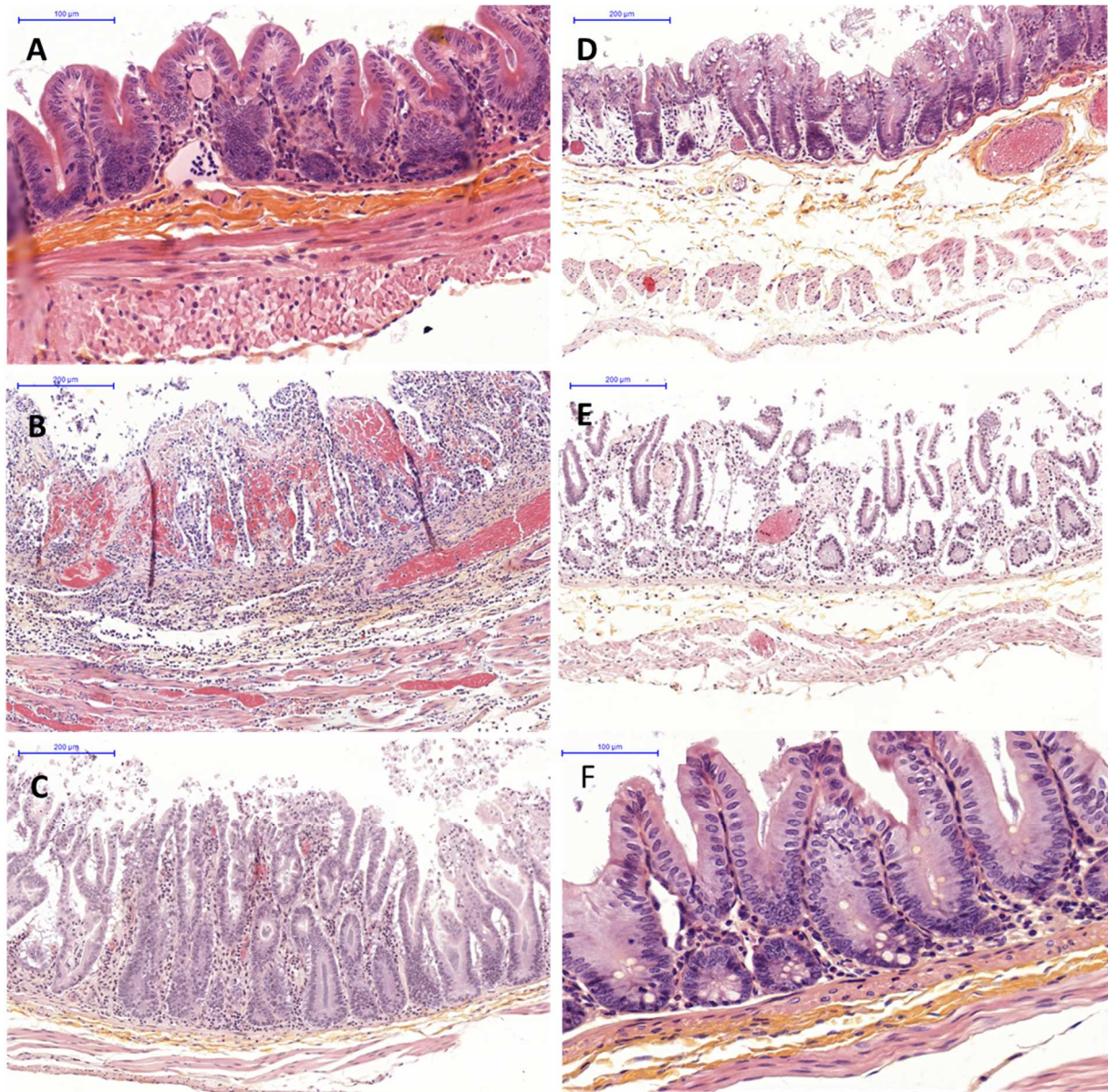


Fig. 3. Cecal histology of hamsters showed significant changes according to different strains used for infection. (A) Cecum of a hamster infected with strain CD15-159 (NT). (B) Cecum of a hamster infected with strain CD10-165 ( $A^-B^+CDT^+$ ). (C) Cecum of a hamster infected with strain CD13-125 ( $A^+B^+CDT^+$ ). (D) Cecum of a hamster infected with strain RA09-070 ( $A^+B^-CDT^-$ ). (E) Cecum of the hamster infected with strain CD13-073 ( $A^-B^-CDT^+$ ) who died at day 4. (F) Cecum of a hamster infected with strain CD13-073 ( $A^-B^-CDT^+$ ) euthanized at day 14. All micrographs are of hematoxylin and eosine–stained tissue at a magnification of x100. Bars represent 100 or 200  $\mu$ m.

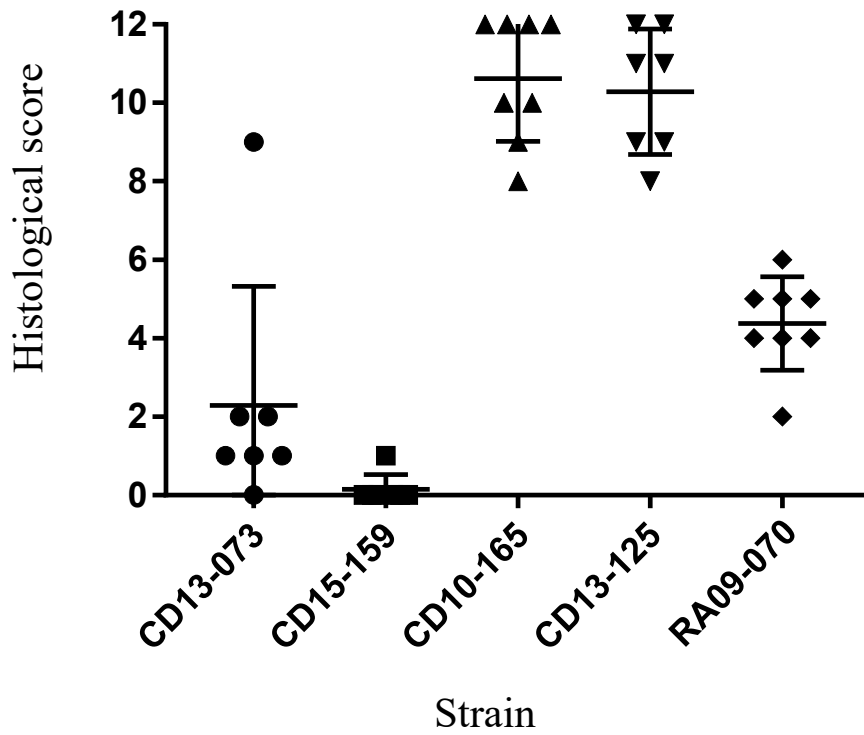


Fig. 4. Histological differences in the ceca of hamsters infected with the various strains of *C. difficile*. The criteria were neutrophil infiltration, haemorrhagic congestion, hyperplasia and percent of epithelial barrier involvements with a maximal score of 12. The bars represent the mean scores for each group of animals and standard deviations.