



## Effect of Antibiotic Therapy on Human Fecal Microbiota and the Relation to the Development of *Clostridium difficile*.

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### ► To cite this version:

Marie-France De La Cochetière, T. Durand, Valérie Lalande, Jean-Claude Petit, Gilles Potel, et al.. Effect of Antibiotic Therapy on Human Fecal Microbiota and the Relation to the Development of *Clostridium difficile*.. *Microbial Ecology*, Springer Verlag, 2008, 56 (3), pp.395-402. <10.1007/s00248-007-9356-5>. <inserm-00286509>

**HAL Id: inserm-00286509**

**<http://www.hal.inserm.fr/inserm-00286509>**

Submitted on 22 Jan 2009

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1 **Effect of antibiotic therapy on human fecal microbiota and the relation to the**  
2 **development of *Clostridium difficile*.**

3  
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17 **Key words:** *Clostridium*, Microbiota, TTGE, PLS regression.

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22 **Running head:** Resident microbiota, *Clostridium difficile*.

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36 **Abstract**

37 The gastrointestinal (GI) tract is a complex ecosystem. Recent studies have shown that the  
38 human fecal microbiota is composed of a consortium of microorganism. It is known that  
39 antibiotic treatment alters the microbiota, facilitating the proliferation of opportunists that  
40 may occupy ecological niches previously unavailable to them. It is therefore important to  
41 characterize resident microbiota to evaluate its latent ability to permit the development of  
42 pathogens such as *Clostridium difficile*. Using samples from 260 subjects enrolled in a  
43 previously published clinical study on antibiotic-associated diarrhea, we investigated the  
44 possible relationship between the fecal dominant resident microbiota and the subsequent  
45 development of *C. difficile*.

46 We used molecular profiling of bacterial 16S rDNA coupled with PLS regression analysis.  
47 Fecal samples were collected on day 0 (D<sub>0</sub>) before antibiotic treatment and on day 14 (D<sub>14</sub>)  
48 after the beginning of the treatment. Fecal DNA was isolated and V6-to-V8 regions of the 16S  
49 rDNA were amplified by PCR with general primers and analyzed by Temporal Temperature  
50 Gradient gel Electrophoresis (TTGE). Main bacteria profiles were compared on the basis of  
51 similarity (Pearson correlation coefficient). The characteristics of the microbiota were  
52 determined using Partial Least Square (PLS) discriminant analysis model.

53 Eighty seven TTGE profiles on D<sub>0</sub> have been analyzed. The banding pattern was complex in  
54 all cases. The subsequent onset of *C. difficile* was not revealed by any clustering of TTGE  
55 profiles, but was explained up to 46% by the corresponding PLS model. Furthermore 6 zones  
56 out of the 438 dispatched from the TTGE profiles by the software, happened to be specific for  
57 the group of patients who acquired *C. difficile*. The first approach in the molecular  
58 phylogenetic analysis showed related sequences to uncultured clones. As for the 87 TTGE  
59 profiles on D<sub>14</sub> no clustering could be found either, but the subsequent onset of *C. difficile*  
60 was explained up to 74.5% by the corresponding PLS model, thus corroborating the results  
61 found on D<sub>0</sub>.

62 The non exhaustive data of the microbiota we found should be taken as the first step to assess  
63 the hypothesis of permissive microbiota. The PLS model was used successfully to predict *C.*  
64 *difficile* development. We found that important criteria in terms of main bacteria could be  
65 markedly considered as predisposing factors for *C. difficile* development. Yet the resident  
66 microbiota in case of Antibiotic-Associated Diarrhea (AAD) has still to be analyzed. Further  
67 more, these findings suggest that strategies reinforcing the ability of the fecal microbiota to  
68 resist to modifications would be of clinical relevance.

69

70 **Introduction**

71 The gastrointestinal (GI) tract is a complex ecosystem generated by the alliance of GI  
72 epithelium, immune cells and resident microbiota. Experimental systems such as cell culture,  
73 germ-free animal models and intestinal isografts have demonstrated that each member of the  
74 GI ecosystem can follow a predetermined developmental pathway, even if isolated from the  
75 other components of the ecosystem. However, the presence of all three components is  
76 required for full physiological function [19]. Genetic or functional alterations of any one  
77 component of this ecosystem can result in a broken alliance and subsequent GI pathology. In  
78 this work, we will focus only on resident microbiota.

79 In both health and disease, the colonic microbiota plays an important role in several areas of  
80 human physiology [15]. But this complex ecosystem is far from well known [29]. Culture-  
81 independent 16S rDNA analyses have previously been used to examine the microbial  
82 diversity of the human gut [25] and explorative multivariable analyses of 16S rDNA data to  
83 study specific microbial communities [23].

84 In a previous study Beaugerie *et al.* clarified the role of *C. difficile* in Antibiotic-Associated  
85 Diarrhea (AAD) in the community by prospectively studying a population of general-practice  
86 patients by means of routine screening for both *C. difficile* and the *C. difficile* toxin B. *C.*  
87 *difficile* was diagnosed by specific culture methods, and *C. difficile* toxin B was detected by  
88 its cytopathic effect. As for diarrhoea, it was scored with the help of validated visual support.  
89 Beaugerie's study was the first to demonstrate a high rate of acquisition (2,7%) of toxin-  
90 producing *C. difficile* during antimicrobial chemotherapy [2].

91 *C. difficile* produces two major toxins (toxins A and B). These are thought to be primarily  
92 responsible for the virulence of the bacterium and the major contributors to the pathogenesis  
93 of antibiotic-associated gastrointestinal disease [4]. Following most antibiotic treatment there  
94 will be a point at which the impact on the normal gut microbiota depresses colonization  
95 resistance to *C. difficile*. The composition of the pre-existing microbiota may have an  
96 important role as well.

97 Therefore, in view of literature data [7, 16, 21, 26], we judged as particularly promising to  
98 investigate the stool of the patients from Beaugerie's previous work. Thus the aim of the  
99 present study was to test the hypothesis of predisposing factors slot in the resident microbiota.

100 We used a genetic fingerprinting method. The characteristics of the microbiota were  
101 determined using Partial Least Square (PLS) discriminant analysis model.

102

## 103 **Methods**

104 **Patients.** Our work is an explicative microbiological approach derived from a clinical study  
105 published elsewhere [2]. In short, 260 subjects enrolled in the latter study were adult out-  
106 patients living in the Paris area, who were prescribed a 5-10 day course of antimicrobial  
107 chemotherapy. Criteria for enrolment were prescription by a general practitioner of a 5-10 day  
108 course of antibiotics and age 18 years or older. Potential candidates were excluded if they  
109 were institutionalized subjects, had received antibiotic treatment during the previous 2  
110 months, had been admitted to a hospital during the previous 6 months, had known human  
111 immunodeficiency virus infection, had any allergy, or had had a bout of diarrhea (more than 2  
112 loose stools per day) the day before enrolment. All patients had given their written consent.  
113 Each patient was asked to store the last stool before the beginning of the antibiotherapy (D<sub>0</sub>),  
114 and the stool 14 days after the beginning of the antibiotherapy (D<sub>14</sub>), in double-thickness  
115 containers, and to keep them in a refrigerator or in a cool place until collection by the study  
116 monitor [2]. The antibiotics given were classified into 3 classes: class 1,  
117 amoxicillin/clavulanic acid; class 2, other beta-lactam agents; class 3, non beta-lactam agents.  
118 Among the 260 patients, 11 acquired *C. difficile*. Among the 249 remaining patients without  
119 *C. difficile*, 38 were chosen because they developed an AAD and paired with patients with no  
120 AAD according to age range (within 10-years) and class of antibiotic. Thus our study  
121 included a total of 87 patients. The 11 patients, 3 men and 8 women, with acquired *C. difficile*  
122 were from 28 to 73 years old, 5 had taken Pristinamycin, 3 of them Amoxicillin, and 3 of  
123 them Amoxicillin/clavulanic acid. Because a typical initial antibiotic dose has no effect on  
124 dominant fecal microbiota for at least 8 to 10 hours (data not shown) we considered the  
125 dominant microbiota profile on D<sub>0</sub> as the profile at equilibrium for each patient.

126

127 **DNA isolation, 16S rDNA amplification.** Stool samples were collected in sterile tubes and  
128 immediately stored at -80°C until analysis. Total DNA was isolated from fecal samples by  
129 using the bead beating method [28]: Immediately after collection, total DNA was extracted  
130 from a 125-mg fecal sample aliquot and purified as described by Godon et al.[10] The DNA  
131 concentration and its integrity (size, >21 kb) were estimated by agarose gel electrophoresis  
132 (with 1.5% [wt/vol] agarose-1× Tris-borate-EDTA-1 ng of ethidium bromide ml<sup>-1</sup>). DNA  
133 was obtained from all samples (0.23 ± 0.1 µg/µl). DNA isolated was subsequently used as a  
134 template to amplify the V6 to V8 regions of the bacterial 16S rDNA with primers U968-GC  
135 (5' CGC CCG GGG CGC GGC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC  
136 GAA GAA CCT TAC) and L1401 (5' GCG TGT GTA CAA GAC CC ). PCR was performed

137 using Hot Star Taq DNA polymerase (Qiagen, Courtaboeuf, France). PCR mixtures of 50 $\mu$ L  
138 contained: 1X PCR Buffer, 1.5 mM Mg Cl<sub>2</sub>, 0.1 mM of each dNTP, 0.5  $\mu$ M of primers U968-  
139 GC and L1401, 2,5 U of Hot Star® Taq Polymerase, and approximately 1 ng of DNA. The  
140 samples were amplified in a Gene Amp PCR system 9700® (Perkin-Elmer, Nantes, France)  
141 by using the following program: 95°C for 15 min; 30 cycles of 94°C for 1min, 56°C for 1min,  
142 72°C for 1.5 min, and finally 72°C for 15 min.

143

144 **TTGE analysis of PCR amplicons** Temporal Temperature Gradient gel Electrophoresis has  
145 been chosen (TTGE) as the culture independent method that allowed the main bacteria  
146 diversity to be compared among samples [27].

147 The Dcode universal mutation detection system (Bio-Rad, Paris, France) was used for  
148 sequence-specific separation of PCR products. Electrophoresis was performed through a 1  
149 mm thick, 16 x16 cm polyacrylamide gel (8% wt/vol acrylamide-bisacrylamide, 7 M urea,  
150 1.25x % Tris-acetate EDTA (TAE), 55  $\mu$ L and 550  $\mu$ l of Temed and ammonium persulfate  
151 10%, respectively) using 7 liters of 1.25x TAE as electrophoresis buffer. Electrophoresis was  
152 run at a fixed voltage of 65 V for 969 min with an initial temperature of 66°C and a ramp rate  
153 of 0.2°C/h. For better resolution, voltage was fixed at 20 V for 5 min at the beginning of  
154 electrophoresis. Each well was loaded with 100-200 ng of amplified DNA plus an equal  
155 volume of 2x gel loading dye (0.05% bromophenol blue, 0.05% xylene cyanol, and 70%  
156 glycerol). As described earlier, a marker was used [7]. A temperature gradient from 66 to  
157 70°C (ramp rate of 0.2°C/hour) was applied during electrophoresis. After completion of  
158 electrophoresis, the gel was stained in a 30  $\mu$ g/mL Sybr Green solution (Sybr Green I, Sigma-  
159 Aldrich, St Quentin Fallavier, France), destained in 1.25x TAE, and analyzed using Quantity  
160 One® software of the Gel Doc 2000® system (Bio-Rad, Paris, France). Profiles were  
161 numerized and gray intensity recorded along a densitogram, each band giving rise to a peak.

162

163 **TTGE gel analysis.** TTGE profiles were compared by using Gel Compare II software  
164 (Applied-Maths, Saint-Martens –Latem, Belgium). The analysis took into account the number  
165 of bands, their position on the gel, and their intensity. This software translates each TTGE  
166 profile into a densitometric curve, drawing a peak for each band (the area under the peak  
167 being proportional to the intensity of the band). A threshold area value was used to remove  
168 small peaks on the densitometric curves (these can be detected purely as a result of the  
169 amount of DNA applied to the gel). A marker consisting of a PCR amplicon mix of seven

170 cloned rDNAs from different bacterial species was used to normalize the profiles. During this  
171 step the gel strips were stretched or shrunk so that the assigned bands on the reference  
172 patterns matched their corresponding reference positions. Similarity coefficients (Pearson  
173 correlation method) were then calculated for each pair of profiles, yielding a similarity matrix.  
174 A dendrogram was constructed from this matrix by using a UPGMA algorithm (unweighted  
175 pair group method using arithmetic averages) [14].

176

177 **Sequence analysis.** Each electrophoregram consisted of a curve in which grey intensity =  
178 f(migration distance). The curves were digitized from the migration distances ranging from 1  
179 to 438 at steps of 1 interval, using the Gel Compare II software. Thus TTGE profiles were  
180 dispatched into zones of interest from which dominant bands were selected and excised for  
181 PCR and sequence analysis. Gel fragments were washed once in 200  $\mu$ L PCR water and kept  
182 in 100  $\mu$ L PCR water overnight at 4°C for diffusion. Ribosomal DNA fragments were then  
183 amplified from the dialyzate. The PCR reaction was as described above. The size and  
184 concentration of the amplicons were evaluated on 1.5% agarose gel containing EtBr. PCR  
185 products were sequenced by Genome Express (Meylan, France). Newly determined sequences  
186 were compared with those in GenBank by BlastN search (NCBI) and using the Ribosomal  
187 Database Project RDP II sequence-match facility (Michigan State University, USA) in order  
188 to ascertain their closest relatives.

189

190 **Multiple linear regression analysis.** PLS-regression (PLSR) is a method for relating two  
191 data matrices, X and Y, by a linear multivariate model, but goes beyond traditional regression  
192 in that it models also the structure of X and Y. PLSR derives its usefulness from its ability to  
193 analyse data with many, noisy, collinear, and even incomplete variables in both X and Y.  
194 PLSR has the desirable property that the precision of the model parameters improves with the  
195 increasing number of relevant variables and observations. In the present study X variables are  
196 the TTGE profiles of dominant resident microbiota and variable Y is the presence or absence  
197 of *C. difficile*. Furthermore, this method allows the assumption that component X (dominant  
198 resident microbiota) is the component that is most relevant for predicting the variable Y  
199 (presence or absence of *C. difficile*) among others (sex, age, antibiotherapy). Relationships  
200 using PLS regression were established between dominant microbiota profiles and status of  
201 patients, using the SIMCA software, version 9.0 (UMETRI, Umeå,  
202 Sweden/www.umetrics.com). Each patient was given a code in which sex, stool sample,  
203 status, antibiotics and age were stated. First the TTGE profiles of each patient on D<sub>0</sub> and D<sub>14</sub>



204 were established. Then they were analysed using the Gel Compare software. Finally, PLS  
205 regression was used to investigate the relations between TTGE profiles of patients (X  
206 variables) and the presence or absence of *C. difficile* (variable Y). The number of useful PLS  
207 components is determined by cross-validation (SIMCA-P 9.0, 2001). The X-loadings and the  
208 Y-loadings are noted  $w^*$  and  $c$ , respectively. Groups of patients are presented as situated on a  
209 plane defined by PLS components. The explanatory performance of the model is evaluated  
210 using the  $R^2$  coefficient which corresponds to the part of the variance of variable Y explained  
211 by the X variables [18].

212

## 213 **Results**

214

215 **Storage of fecal samples.** In the previously published clinical study the interval between stool  
216 passage and laboratory processing was  $26.9 \pm 8.3$  h (range, 3 to 71 h). In order to test the  
217 stability of the dominant fecal microbiota, fecal samples from 4 healthy volunteers were  
218 analysed in parallel. Those samples were kept simultaneously at  $-80^\circ\text{C}$ ,  $4^\circ\text{C}$ , and  $20^\circ\text{C}$  for 24,  
219 48 and 72 hours and then analyzed by Temporal Temperature Gradient gel Electrophoresis  
220 (TTGE). Results are expressed as percentages of similarity of TTGE profiles after storage  
221 under the indicated conditions in comparison with an aliquot of the same sample stored at -  
222  $80^\circ\text{C}$  (as the gold standard). After 24 hours, the percentages of similarity of TTGE profiles  
223 were of  $88.6\% \pm 5.2$  with storage at  $4^\circ\text{C}$  and  $89.2\% \pm 1.8$  with storage at  $20^\circ\text{C}$ . After 48 hours  
224 they were  $89.1\% \pm 4$  and  $87.5\% \pm 1.9$  respectively and after 72 hours they were  $82\% \pm 7.4$   
225 and  $86\% \pm 3.3$  respectively.

226

227 **Intra-individual analysis.** 174 TTGE profiles were analyzed, from  $D_0$  and  $D_{14}$  for each of the  
228 87 patients studied. The banding pattern was complex in all cases. The dendrogram analysis  
229 showed that the TTGE profiles did not cluster according to presence or absence of *C. difficile*  
230 (UPGMA dendrogram not shown). Similarity percentages between  $D_0$  and  $D_{14}$  profiles for  
231 each of the 11 patients with acquired *C. difficile*, according to antibiotic classes, varied from i)  
232 0% (patient with AAD) and 90.8% (patient without AAD) for amoxicillin – clavulanic acid,  
233 ii) 41% (patient with AAD) and 71.3% (patient without AAD) for other beta-lactam agents  
234 and iii) 60.4% (patient with AAD) and 84.4% (patient without AAD) for non beta-lactam  
235 antibiotics.

236

237 **Inter-individual analysis and PLS model:** Relationships between the profiles included in the  
238 PLS model and the results of “acquired *C. difficile*” are not easily established using a visual  
239 observation of the profiles. We calculated a PLS model that linked the 87 TTGE profiles of  
240 main bacteria (X variables) of patients and the subsequent onset of *C. difficile* (variable Y),  
241 first on D<sub>0</sub> then on D<sub>14</sub>. The cross-validation led to R<sup>2</sup> coefficient which denotes the  
242 percentage of variation. On D<sub>0</sub> the corresponding PLS model explained 46% of the variation  
243 of the Y-matrix (development of *C. difficile*) (Fig. 1) and on D<sub>14</sub> 74.5% (Fig. 2).

244  
245 **Sequence analysis:** D<sub>0</sub> TTGE profiles of the 11 patients with acquired *C. difficile* were  
246 compared on the same gel (Fig. 3). The analysis of the 438 zones dispatched from TTGE  
247 profiles by the software, showed that only 6 were specific for the group of patients with  
248 acquired *C. difficile*. From those 6 zones of interest, 7 main bands have been selected taking  
249 into account their optical density and distinctiveness. To gain insight into the phylogenetic  
250 positions of those amplicon DNA, they were extracted from the gel and sequenced. The  
251 sequences showed the highest similarity with sequences derived from different *Clostridium*  
252 clusters of the low guanine+cytosine (G+C) gram positive species [5]. The average  
253 determined length of the DNA sequences was 500 bases, and phylogenetic analysis was based  
254 on 400 to 450 aligned homologous nucleotides (corresponding to positions 900 to 1400 in  
255 *Escherichia coli* 16S rDNA). Using the same approach, 2 zones of interest were identified at  
256 D<sub>14</sub>. Three bands were selected, extracted and sequenced. The characteristics of the 7  
257 extracted sequences of D<sub>0</sub> and of the 3 extracted sequences of D<sub>14</sub> are shown in table 1 with  
258 the origin (fecal sample), sequence length (370-417), closest relative identification and  
259 percentage of identification. They all belong to Clostridiales order, Clostridiaceae,  
260 Eubacterium and Lachnospiraceae family.

261

## 262 **Discussion**

263 Our results support the concept of “permissive” microbiota. Using molecular profiling of  
264 bacterial 16S rDNA coupled with PLS regression analysis, we found that important criteria in  
265 terms of main bacteria of the fecal microbiota could be considered as predisposing factors for  
266 *C. difficile* development. We did not intend to sequence all implicate bacteria species. The  
267 studied patients were adult out-patients living in the Paris area. We assumed that all were  
268 exposed equally to *C. difficile* from the environment. The PLS regression analysis gave a  
269 predictive ability of 46% for the resident microbiota of those patients who developed a  
270 *C.difficile* after an antibiotherapy. These results explain the development of *C.difficile*

271 following antibiotic treatment and corroborate a metaproteomic approach to link biological  
272 functions to gene sequences. Furthermore, these findings suggest that strategies reinforcing  
273 the ability of the resident microbiota to resist to modifications would be of clinical relevance.  
274 Recent culture-independent molecular studies on healthy individuals have shown that the  
275 intestinal microbiota is specific to the host and resistant to modification over time [27].  
276 Although, the difficulty to identify the exact profile at equilibrium has already been discussed  
277 [6], to take into account every patient's data including factors such as age, sex, clinical and/or  
278 antibiotherapy, is rather difficult. It needs the help of an abstract model that uses  
279 mathematical language to describe the behavior of the system by a set of variables and a set of  
280 equations that establish relationships between the variables. PLS-regression is a particular  
281 type of multivariate analysis which uses the two-block predictive PLS model to model the  
282 relationship between two matrices. PLS-regression derives its usefulness from its ability to  
283 analyze data with many, noisy, collinear, and even incomplete variables in both X and Y.  
284 PLS-regression has the advantageous property that the precision of the model parameters  
285 improves with the increasing number of relevant variables and observations [8]. Thus we  
286 chose PLS-regression for relating the resident microbiota to *C. difficile* development. PLS-  
287 regression has been used in various disciplines such as chemistry, economics, medicine,  
288 pharmaceutical science and microbiology [13, 18, 20]. The PLS-regression analysis of the  
289 resident microbiota on D<sub>0</sub> gives one significant component explaining 46% of the Y-variance.  
290 Our analysis shows that the data are clustered: The resident microbiota from patients with  
291 later *C. difficile* development deviates from the main cluster. In addition PLS-regression  
292 detected the variable that is highly linked to variable Y (acquired *C. difficile*) among a large  
293 number of X variables (TTGE profile, age, sex and class of antibiotic) as applied to a large  
294 number of observations. In our model the variable X (=TTGE profiles) had been detected  
295 based on its significance, other X variables (age, sex and class of antibiotic) were not relevant  
296 although a specific study with the different antibiotics would be warranted.  
297 Thus the present report provides evidence for predisposing factors in resident microbiota.  
298 Such scoring functions should aid in the identification of putative group of bacteria. Moreover  
299 the model could be used to predict the inclusion of new patients by incorporating their TTGE  
300 profiles into the model. Therefore, more information could be obtained from TTGE profiles  
301 than those given by densitometric analyses. This model enabled the parameters affecting the  
302 distribution of the microbiota to be examined. Nevertheless the molecular determinants and  
303 host specificity have yet to be identified.

304 Operational Taxonomic Unit or molecular species is defined as a set of sequences with less  
305 than 2% divergence in 400-450 aligned homologous nucleotides [25]. Thus most of the  
306 sequences identified in this work were related to uncultured bacterium clones (99-98%) from  
307 *Clostridiales* order. Among the anaerobes the *Clostridiales* order are known to have a strong  
308 catalytic activity.

309 The non exhaustive data of the microbiota we found show species only from *Clostridiales*  
310 order, *Clostridiaceae*, *Eubacterium* and *Lachnospiraceae* family. This is not surprising since  
311 novel or yet uncultured species are most often identified upon characterization of fecal  
312 microbiota using cloned 16S rDNA genes libraries [11]. Recent culture-independent studies  
313 have shown that approximately 70% of the dominant human gut microorganisms have not  
314 been isolated and described [3].

315 Interestingly, after subtractive densitometric analyses on D<sub>0</sub> for the selected band 1, we  
316 found that it was common in 10 out of the 11 TTGE profiles of the selected patients and only  
317 2 out of the 76 others. Furthermore, among the 11 patients with acquired *C. difficile*, 5  
318 developed AAD. They clustered separately from the 6 patients without AAD on D<sub>0</sub> as well as  
319 on D<sub>14</sub>, suggesting two different “sub-groups” of dominant microbiota. These results’  
320 analyses are consistent with our hypothesis and merit confirmation. This will be tested with  
321 patients who developed AAD.

322 Within D<sub>14</sub> of antibiotherapy, the human fecal microbiota of patients was markedly  
323 modulated. The alterations observed here (0% patient with AAD and 90.8% patient without  
324 AAD) in the structure of the microbiota upon amoxicillin – clavulanic acid treatment are  
325 important enough to suggest two groups of patients. It had been determined that upon natural  
326 oscillations of dominant fecal microbiota TTGE profiles would remain within 90% of  
327 similarity with the equilibrium state over a period of two years in one volunteer [24]. Thus,  
328 these observations suggest the occurrence of a specific resistant microbiota to amoxicillin –  
329 clavulanic acid and would warrant confirmation.

330 From the 5 patients with AAD one was found neither toxinogenic nor with toxin. Thus, in this  
331 case, *C. difficile* could not be considered as cause of diarrhea, although a negative search for  
332 toxin and/or toxinogenesis obviously does not constitute final proof for the absolute lack of *C.*  
333 *difficile* spore in the microbiota.

334 The dendrogram analysis showed that the TTGE profiles did not cluster according to presence  
335 or absence of *C. difficile*. But the PLS –regression model explained 46% of the variation of  
336 the Y-matrix (development of *C.difficile*). This study assessed for each individual, the  
337 significance of resident microbiota but did not intend to determine the composition of the

338 dominant fecal microbiota in terms of bacterial genera or species. The new sequences found  
339 in the genus *Clostridium* indicate the importance of this genus inside the microbiota and its  
340 putative role in development of pathogens [7]. Molecular analyses of the bacterial microbiota  
341 based on 16S rDNA have attracted attention as reliable methods for detection and  
342 identification of bacterial species [1, 12, 17]. Techniques such as temporal temperature  
343 gradient gel electrophoresis are attractive because they are conducive to high throughput  
344 studies. TTGE successfully differentiates bacterial gene fragments of the same size but  
345 different thermal stability. The uses and limits of TTGE in microbial ecology have already  
346 been explored [7, 22]. The ability to apply statistical methods makes denaturing gel  
347 electrophoresis fingerprinting techniques such as TTGE tools with great potential [7, 9]. Only  
348 the dominant fraction of the fecal microbiota is assessed using the PCR-TTGE technique, as  
349 applied here, with universal primers. The complexity of the profiles observed by TTGE will  
350 represent the most prevalent species.

351

352

353

354

355 **Acknowledgments:** We express our gratitude to Dr. P. Tailliez and Pr. A. Andreumont for  
356 their helpful advice. Mr. T. Durand was supported by a grant from Biocodex Inc.

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**Table 1:** Data of the sequences: origin, sequence length, closest relative identification (accession number), % identity.

<b>N° extract. bande</b>	<b>Accesion Number</b>	<b>Origin</b>	<b>Sequence length (letter)</b>	<b>Closest relative</b>	<b>Phylum/class of the closest relative</b>	<b>% Identity</b>
1	EU196222	Fecal sample Individual	395	Uncultured bacterium	Genus Ruminococcus, (DQ802748)	99
2	EU196223	Fecal sample Individual	394	Uncultured bacterium	Unclassified_Clostridiales (AY984391)	99
3	EU196224	Fecal sample Individual	395	Clostridium	Clostridium sp. (AJ582080)	98
4	EU196225	Fecal sample Individual	391	Uncultured bacterium	Genus Ruminococcus (DQ905852)	99
5	EU196226	Fecal sample Individual	370	Uncultured bacterium	Genus Ruminococcus (AM277309)	99
6	EU196227	Fecal sample Individual	386	Uncultured bacterium	Genus Dorea/ family Clostridiaceae (DQ802652)	99
7	EU196228	Fecal sample Individual	394	Uncultured bacterium	Genus Eubacterium (AM275432)	91
8	EU196229	Fecal sample Individual	405	Uncultured bacterium	unclassified_Lachnospiraceae (AY235653)	100
9	EU196230	Fecal sample Individual	417	Uncultured bacterium	Genus Ruminococcus	
10	EU196231	Fecal sample Individual	389	Uncultured bacterium	Genus Ruminococcus Uncultured Firmicutes (EF071261)	94

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459 **Legends to figures**

460 **Figure 1:** 3D representation of the PLS regression model showing relationship between  
461 TTGE profiles of dominant species before any antibiotic treatment ( $D_0$ ) and the subsequent  
462 onset of *C. difficile*. The corresponding model explained 46% of the estimated modification.  
463 Positions of the  $D_0$  TTGE profiles of patients who acquired *C. difficile* are indicated in this  
464 3D representation, by rectangles, position of the  $D_0$  TTGE profiles of others are indicated by  
465 triangles.

466

467 **Figure 2:** 3D representation of the PLS regression model showing relationship between  
468 TTGE profiles of dominant species 14 days after the antibiotic treatment ( $D_{14}$ ) and the  
469 subsequent onset of *C. difficile*. The corresponding model explained 74.5% of the estimated  
470 modification. Positions of the  $D_{14}$  TTGE profiles of patients who acquired *C. difficile* are  
471 indicated in this 3D representation, by rectangles, position of the  $D_{14}$  TTGE profiles of others  
472 are indicated by triangles.

473

474 **Figure 3:** Temporal temperature gradient gel electrophoresis of 16S rDNA amplicons  
475 (amplified with universal primers for the V6-V8 region of the gene) of fecal samples obtained  
476 at day 0, before any antibiotherapy, from the 11 patients who acquired a *C. difficile* after the  
477 antibiotherapy.

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479 **Figure 4:** Example of Temporal temperature gradient gel electrophoresis of 16S rDNA  
480 amplicons (amplified with universal primers for the V6-V8 region of the gene) of fecal  
481 samples from patients 6 and 9, who acquired *C. difficile* after the antibiotherapy, at Day 0 and  
482 Day 14.

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493 **Figure 1:**

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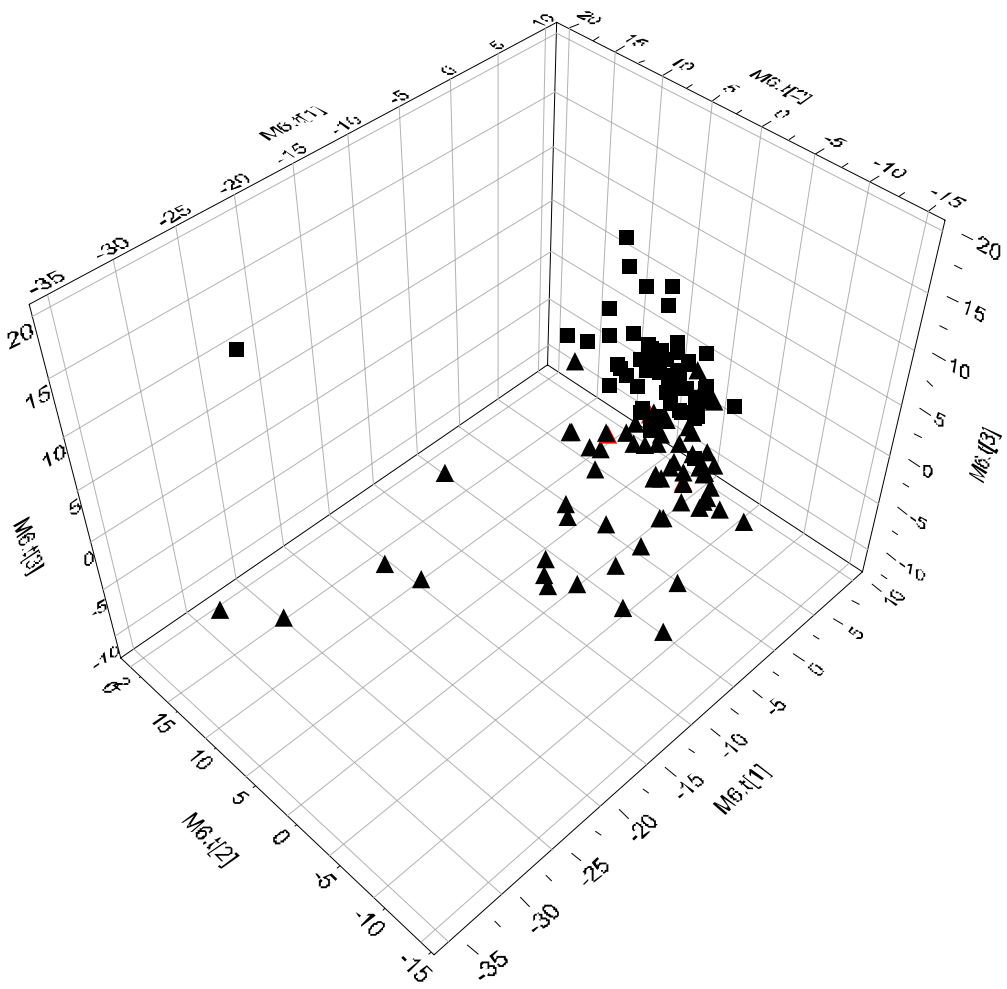
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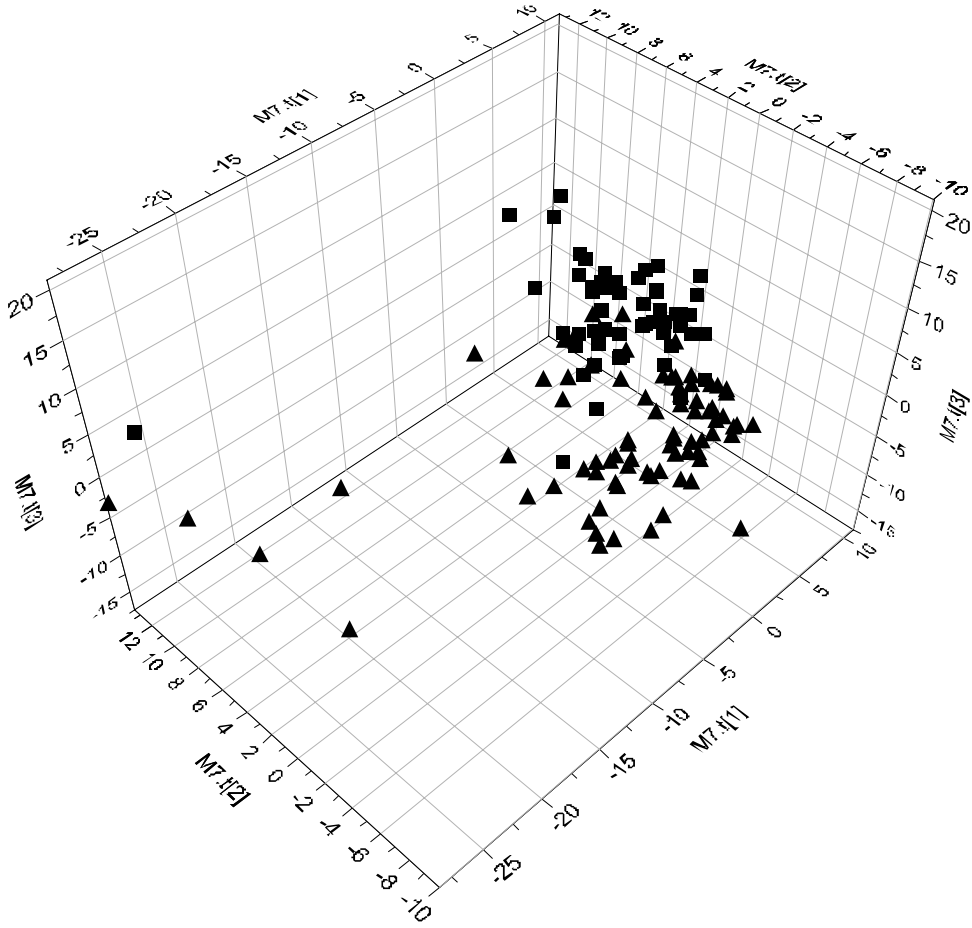
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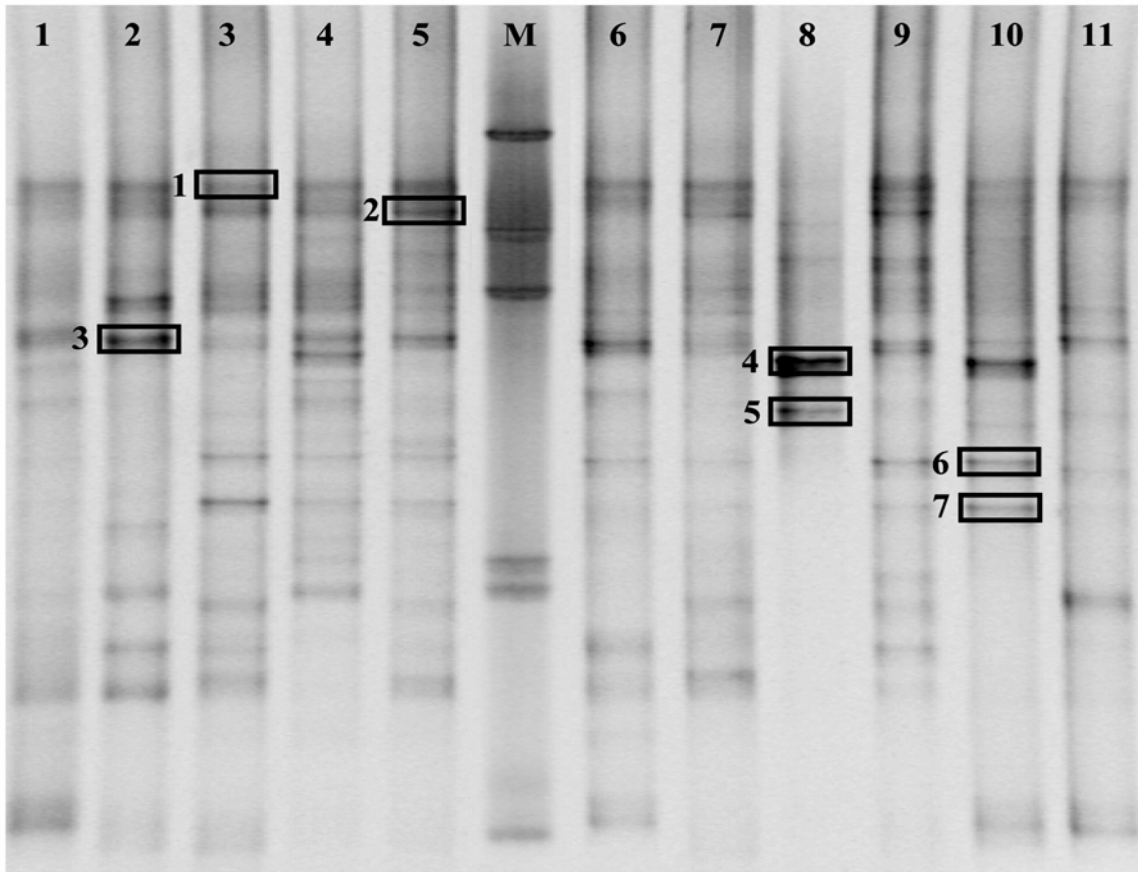


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561 **Figure 3:**

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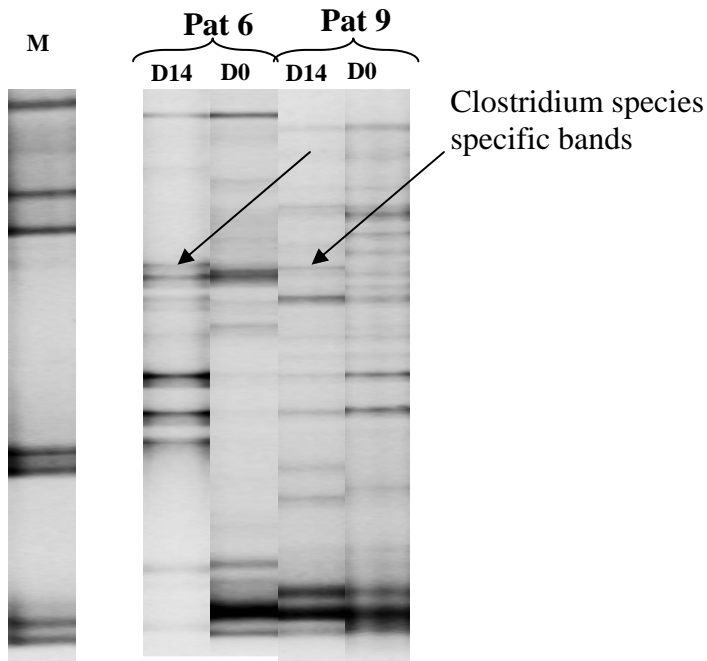
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566 **Figure 4:**  
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