Human intestinal microbiota gene risk factors for antibiotic-associated diarrhea: perspectives for prevention. Risk factors for antibiotic-associated diarrhea.

Marie France De La Cochetière, Emmanuel Montassier, Jean-Benoît Hardouin, Thomas Carton, Françoise Le Vacon, Tony Durand, Valérie Lalande, Jean-Claude Petit, Gilles Potel, Laurent Beaugerie

Human intestinal microbiota gene risk factors for antibiotic-associated diarrhea: perspectives for prevention

Marie-France de La Cochetière[1]*, Emmanuel Montassier[4], Jean-Benoit Hardouin[5], Thomas Carton[4], Françoise Le Vacon[3], Tony Durand[4], Valerie Lalande[2], Jean Claude Petit[2], Gilles Potel[4], Laurent Beaugerie[2].

[1]**INSERM, Université de Nantes, UFR Médecine**, Thérapeutiques Cliniques et Expérimentales des Infections, EA 3826, rue G. Veil, Nantes, F-44000 France.

[2]**Service de Gastro-entérologie et Nutrition, Hôpital Saint-Antoine**, 184 rue du faubourg Saint-Antoine, 75012 Paris Cedex, France.

[3]**Atlangene®-Silliker**, Bio Ouest, Ile de Nantes, 21 rue La Noue Bras de Fer, 44200 Nantes, France

[4]**Université de Nantes, UFR Médecine**, Thérapeutiques Cliniques et Expérimentales des Infections, EA 3826, rue G. Veil, Nantes, F-44000 France.

[5] **Université de Nantes, UFR Médecine et Pharmacie,** Biostatistics Clinical Research and Subjective Measures in Health Sciences , EA 4275, rue G. Veil, Nantes, F-44000 France.

Running title: Diarrhea risk prediction from microbiota genes

*__Corresponding author__ Dr. MF de La Cochetière Mailing address: UPRES EA 3826, UFR de Médecine, 1 rue Gaston Veil, 44035 Nantes, cedex 01, France. Phone 33 (0)240412840, Fax 33(0)240412854. E-mail marie-france.de-la-cochetiere@inserm.fr

Financial support: Biocodex Inc

Abstract

Antibiotic-associated diarrhea (AAD) is associated with altered intestinal microflora and other symptoms that may lead to possibly death. In critically ill patients, diarrhea increases rates of morbimortality. Assessing diarrhea risks is thus important for clinicians. For this reason, we conducted a hypothesis-generating study focused on antibiotic-associated diarrhea (AAD) to provide insight into methods of prevention. We evaluated the hypothesis of predisposing factors within the resident intestinal microbiota in a cohort of outpatients receiving antibiotherapy. Among the pool of tested variables, only those related to bacterial 16S rRNA genes were found to be relevant. Complex statistical analyses provided further information: amid the bacteria 16S rRNA genes, eight were determined to be essential for diarrhea predisposition and characterized from the most important to the least. Using these markers, AAD risk could be estimated with an error of 2%. This molecular analysis offers new perspectives for clinical applications at the level of prevention.

Key words: Data-Mining/Diarrhea/Microbiota genes/Prevention/Risk factors

Introduction

The collective effects of the intestinal microbiota are dictated by a complex network of interactions that span the cellular, immunological, and environmental levels. A primary question is whether a simple system can be applied to predict and control the effects of this heterogeneous population composed of different subgroups. One approach in addressing this question is to study representative genes that correlate with the health consequences of the bacteria mixture comprising the microbiota. The present study concerns specific bacterial populations associated with antibiotic-associated diarrhea (AAD). This hypothesis-generating experiment was conducted to discern whether an analysis of bacterial 16S rRNA genes from pre-antibiotic resident faecal microbiota using with complex statistics could predict the collective effects of the intestinal microbiota, thereby identifying individual risk factors for diarrhea associated with antibiotic treatment. These specific gene sequences have been chosen because they have been the far most common genetic marker used (16).

Antibiotic-associated diarrhea (AAD) is associated with altered intestinal microflora, mucosal integrity, vitamin, mineral metabolism and crampy abdominal pain. If severe, AAD may lead to electrolyte disturbances, dehydration, premature discontinuation of antibiotic therapy, pseudomembranous colitis, toxic megacolon and possibly death. Reports in the general population indicate that the incidence of AAD ranges from 25 to 62%, occurring at any point from the initiation of therapy to two months after the end of treatment (2, 17). In critically ill patients, diarrhea increases morbimortabilty. AAD leads also to longer hospital stays and higher medical costs (1, 26, 27). The pathogenesis of AAD may be mediated through the disruption of the normal microbiota and overgrowth of pathogens, or through metabolic imbalances (3, 31). The individual risk for AAD varies greatly, influenced by host factors (age or diet), and type, dose, and duration of antibiotherapy (15, 22). Assessment of

87 the diarrhea risk during antibiotic treatment is therefore worthwhile and may help control
88 diarrhea in defined high-risk individuals; however, few data exist on risk factors for AAD.
89 The role of the human intestinal microbiota in health and specific diseases is a
90 particularly important area of research (7, 12, 13). Substantial progress has recently been
91 made in characterizing the human intestinal microbiota, although its role in immune system
92 development and regulation, nutrition, and pathogenesis of the host are still not well
93 elucidated (30). Furthermore, the rapid rate of microbial evolution, combined with the global
94 rise of antimicrobial resistance and the low rate of novel antibiotic development underscores
95 the urgent need for innovative therapeutics (5, 10). Culture-based techniques have
96 traditionally been used to determine the faecal microbiota. However, molecular techniques
97 based on analysis of 16S rRNA genes directly amplified from bacterial DNA extracted from
98 feces have estimated that less than 25% of the faecal bacterial populations have been cultured
99 to date (11, 28). These approaches have provided considerable data about microbial
100 ecosystems, including that of the human gastrointestinal tract. Detailed phylogenetic
101 informations have been obtained by cloning and sequencing 16S rRNA genes. Further,
102 several studies with fingerprinting of 16S rRNA genes have reported its benefits in
103 monitoring community shifts (8, 19, 23, 29).
104 Hence, as a system model for the analysis of heterogeneous populations of bacteria,
105 we analysed the 16S rRNA genes in the genomes of all bacteria using temporal temperature
106 gradient gel electrophoresis (6), and multivariable data analysis (21). In this retrospective
107 study, we hypothesized that the susceptibility to diarrhea may be linked to the resident
108 intestinal microbiota. For this purpose we focused on a published clinical study (4), which
109 included epidemiology forms, patient history, and laboratory reports; we analyzed database
110 case records, specimen collection, and risk factors. We designed complex statistical analyses

111 to determine the optimal procedures for providing the maximum relevant information with
112 fingerprint data, and obtaining knowledge about the 16S rRNA gene system.
113
114 **Methods**
115 ***Patients.*** Subjects enrolled in this study were adults (age range, 20-60 years) living in
116 the Paris area. Subjects were prescribed a course of antimicrobial therapy for an ear, nose, or
117 throat infection. Criteria for enrollment included prescription by a general practitioner for a
118 5-to 10-day course of antibiotics and age of 18 years or older. Potential candidates were
119 excluded if they were institutionalized, had received antibiotic treatment during the previous 2
120 months, had been admitted to a hospital during the previous 6 months, had a known human
121 immunodeficiency virus infection, had any allergy, or had experienced a bout of diarrhea (>2
122 loose stools/day) the day before enrollment. All patients provided informed written consent.
123 Prescribed antibiotics were classified into one of the following groups: amoxicillin/clavulanic
124 acid, other beta-lactam agents, or non-beta-lactam agents. Diarrhea was defined as the
125 passage of at least three loose stools a day**,** AAD was defined as diarrhea associated with the
126 administration of antibiotics (in the absence of any other obvious reason) during the 14-day
127 study period. Each patient was asked to store the last stool before the beginning of the
128 antibiotherapy (D_0) in a double-thickness container. The dominant microbiota profile at D_0
129 was considered to be patient's profile at equilibrium (i.e., the resident microbiota). In
130 addition to the molecular study, bacteriological investigations were performed according to
131 standardized procedures.
132 ***DNA isolation and 16S rRNA gene amplification.*** Immediately after faecal sample
133 collection, total DNA was extracted from a 125-mg aliquot and purified as previously
134 described (9). DNA (0.23 ± 0.1 µg/µl) was obtained from all samples. The DNA integrity
135 and concentration (size, >21kb) were determined by 1.5% agarose gel electrophoresis with

ethidium bromide. Isolated DNA was subsequently used as a template to amplify the V6 to V8 regions of the bacterial 16S rRNA gene with primers U968-GC and L1401 (32). Contamination and amplification controls were performed at each steps as previously described (9).

TTGE analysis of PCR amplicons. The Dcode universal mutation detection system (Bio-Rad, Paris, France) was used for sequence-specific separation of PCR products as previously described. After electrophoresis, the gel was stained and analyzed using Quantity One software of the Gel Doc 2000 gel documentation system (Bio-Rad, Paris, France).

TTGE gel analysis. Each grey band of the TTGE gels was considered an amplicon of the 16S rRNA gene. To simplify analysis, we used zones of the electrophoretic gels to describe the migration distances representing individual 16S rRNA genes. TTGE profiles were compared by using Gel Compare II software (Applied-Maths, Sint-Martens–Latem, Belgium). The analysis took into account the number of bands, their positions on the gel, and their intensities. Gray intensities were then recorded along a densitogram, with each band given rise to a distinct peak. Thus each electrophoresis pattern was represented by a curve defined as grey intensity = function (normalized migration distance). A marker consisting of a mixture of PCR amplicons (seven cloned 16S rRNA genes from different bacterial species) was used to normalize the profiles as previously described (9). Similarity coefficients (Pearson correlation method) were calculated for each profile, yielding a similarity matrix. A dendrogram was constructed from this matrix using the UPGMA algorithm (unweighted pair group method using arithmetic averages).

***Data collection*.** The acquired data from all D_0 electrophoresis gels were collected as 147 retention times that resulted from digitizing each electrophoresis at equal distance intervals. Each patient was also characterized by qualitative features related to age, sex,

therapy and the eventual presence of AAD. The ages were recoded into seven classes (from ages 20-29 to 80-89). AAD was represented by two groups (absence or presence).

Multivariable data-analysis

Two analyses of the data has been carried out in order to detect retentions times predictive of the AAD: ANOVA which compare the mean values at each retention times between the two groups, and discriminant analysis which allows to detect the more important retention times to predict AAD.

Analysis of variances. Each of the 147 retention times was explained by a four-way analysis of variance (ANOVA) including age, sex, therapy and AAD as independent variables. The aim of these analysis was to detect the retention times where there was significant differences between the two groups of patients (absence or presence of AAD), by adjusting the analysis on clinical variables (age, sex and therapy).

In order to take into account the multiplicity of the tests, the F statistics associated to absence/presence of AAD of the ANOVA were compared to the value obtained with the following process: 10000 simulated datasets were generated by using the observed retention times and by simulating the factors age, sex, therapy and presence/absence of AAD with the same distributions than these ones observed in the sample independently of the retention times. The retained threshold for the F statistics (referenced as threshold F value) was the value of the F where 5% of the simulated datasets were above. This threshold corresponds to the 5% significant level obtained by chance. Only the retention times where the corresponding F values are above this threshold are retained as significantly predictive of the AAD.

Discriminant analysis. A discriminant analysis was carried out in order to detect retention times which allow predicting the AAD for the patients. Forward selection of the retention times was realized: At each step, the retention time which allows improving the

more the clustering of the patients in the two groups (correct clustering between absence/presence of AAD) is introduced in the analysis, until there is no more possible improvement of the clustering.

Results

Among the 156 patients included in the study, 44 developed an AAD. None of the 44 patients with diarrhea had stool culture positive for the tested intestinal pathogens *Salmonella* spp., *Shigella* spp., *Campylobacter* spp. and *Yersinia* spp.); however, six patients acquired *Clostridium difficile*.

TTGE gel analysis. From DNA extracted from D0 stool samples (before antibiotherapy) 156 TTGE profiles were analyzed. The banding pattern was complex in all cases (Figure 1), the number of bands ranged from 10 to 20. The dendrogram analysis (unweighted pair group method with arithmetic mean (UPGMA) dendrogram not presented here) showed that the TTGE profiles did not cluster according to the onset of AAD. These results corroborate that, for each individual, microbial diversity and composition are specific traits (20). Thus, the need for extra complex analyses is pointed out in order to find any grouping within the microbiota profiles, before treatment.

Multivariable data-analysis: Using multivariate data analysis techniques to determine potential causal factors for AAD, we found that only D_0 microbiota profiles were strongly correlated with AAD. In this study, other factors (age, sex, and class of antibiotic) were not relevant, thus we focused on the intestinal bacterial 16S rRNA genes: each TTGE profile was converted into a curve, then digitized and further analysed to convert 16S rRNA genes to numerical data (see complementary material). We applied advanced multivariate analysis to confirm any predictive signature of the intestinal microbiota.

Variance analysis. Figure 2 shows the *F*- value of ANOVA carried out independently at each data point of each electrophoresis at D_0. The studied factor was the presence/absence of AAD after antibiotherapy. Six electrophoresis migration distance values were found exceed the threshold *F*-value: (A) 58, (B) 70, (C) 174, (D) 321, (E) 358 and (F) 399. Figure 3 shows the mean electrophoresis migration patterns associated with the absence and presence of AAD: significant regions are emphasized using a grey color. Zones A and F are correlated to the microbiota of patients with AAD, and zones B, C, D, and E are correlated to the microbiota of patients without AAD.

Discriminant analysis. Six retention times explaining the absence/presence of AAD have been selected by the forward selection of the variables for the discriminant analysis (FLDA). These 6 variables allow clustering 142 among the 156 patients (91%) in the correct group.

Individual risk prediction of AAD from resident intestinal microbiota. Figure 4 demonstrates the degree of agreement between ANOVA and FLDA. Four of the first six variables selected by the discriminant analysis were also identified by ANOVA, producing distinct profiles for AAD and no AAD. The risk prediction for any new case of AAD could be calculated from its intestinal microbiota gene fingerprinting with an error of 2% in the case of AAD (1/44) and 11% (12/112) in the case of no AAD.

Discussion

Predicting and controlling the effects of a heterogeneous bacteria population is a highly challenging task with many biological and clinical applications. To study this question, we evaluated relationships between intestinal bacterial 16S rRNA genes and AAD, which is a growing health concern. Why some patients develop AAD while others do not is a recurrent and unresolved question. This paper describes for the first time a computational

approach that accurately predicts the relationship between the resident microbiota and the risk for developing AAD.

The important findings of this study are as follows:

i.) Among the studied patient variables (antibiotherapy, age, sex, bacterial 16S rRNA genes), only the pre-antibiotic resident faecal bacterial 16S rRNA genes were found to be correlated to AAD.

ii.) Among the bacterial 16S rRNA genes studied, one group was found to be crucial for the predisposition for AAD. This group is composed of eight specific electrophoretic distances, thus eight specific set of genes, classified from most important to least important: all eight were required for the development of diarrhea.

iii.) Risk factors could be calculated; AAD could be predicted from the resident intestinal bacterial 16S rRNA gene analysis with an error of 2%, and no AAD with an error of 11%.

These observations are based on the investigation of the dominant faecal bacterial populations before any antibiotherapy, by fingerprinting techniques applied to samples from a cohort of outpatients treated by antibiotherapy for ear, nose and throat infections (non invasive sampling). Those conclusions are valid within the context of the study, but do not account for variation outside the dataset.

The strengths of this study include the uniqueness of this type of analysis on bacterial genes from the resident faecal microbiota. It is based on a comprehensive causal model that describes the relationships among numerous risk factors for AAD. The weakness of this study is the lack of phylogenetic analysis; the study does not provide information regarding the specific microorganisms involved in AAD risk. Our aim was to determine the presence of risk factors for AAD among the 16S rRNA genes. The 16S rRNA gene is an ~1500 base pair

gene that codes for a portion of the 30S ribosome. Partial (500-base pair) 16S rRNA gene sequencing has emerged as an accurate method to identify a wide variety of bacteria and has been successfully implemented in clinical laboratories (24, 25). A major limitation of the 16S rRNA gene sequencing, though, is its inability to discriminate among all bacterial taxa. In that case alternative gene targets can provide better separation of closely related species (e.g. *rpoB* gene) (18) . However, in this work, the aim was not to identify bacteria, although it looks rather frustrating not to. Ultimately, the goal is to associate differences in communities with differences in metabolic function and/or disease (AAD). Thus these work and the results explain here, stand as one first step toward it. Therefore, the findings of this study are being used for further work on the impact of the resident microbiota. Then, in-depth phylogenetic analysis of the microbiota will be needed and preventing strategies developed.

We started with the assumption of a predictive signature of the microbiota. The statistical analysis shows significant differences in the migration patterns between the two groups (absence/presence of AAD). Moreover, this analysis shows that the AAD can be correctly predicted with data based exclusively on migration distances. As a consequence, it can be conclude that the nature of the microbiota before antibiotherapy may play a role in AAD.

The variance analysis identified six significant zones (A to F); the discriminant analysis also identified six significant regions (1 to 6) in order of decreasing importance, all of which are required for prediction. Interestingly, only four zones were common between the two analyses (B to 4, C to 2, E to 6 and F to 1). Further, if an intestinal microbiota 16S rRNA gene profile shows the six zones indicating no AAD and does not show the two zones indicating AAD, it can be assumed that the patient is not likely to develop AAD. One potentially confusing aspect of this study is that we speak of numerical data, so we describe risk in terms of “zones”, that is to say, electrophoretic distances representing the16S rRNA

genes. The next area of study will be to determine to which extend they are associated to phylogenetic species.

Fingerprinting techniques such as TTGE are powerful analysis tools for detecting biomedically relevant markers such as nucleic acids and proteins and ultimately diseases or disease progression that can alter the structure of biological systems like intestinal microbiota. Multivariate data-analysis techniques are essential to manipulate and interpret these enormous amounts of data, and appropriately address the inherent complexity of data derived from biomedical samples. In addition, different multivariate algorithms must be tested to determine the most suitable method(s) for establishing reliable, robust, and accurate classification or regression models, while minimizing false-positive and false-negative results. Nonetheless, multivariate data-analysis techniques should be used cautiously, as a complement to optimized diagnostic techniques that already provide relevant information. Specifically, useful information obtained by fingerprinting techniques like TTGE (i.e., bacterial diversity) increases with a priori knowledge of the samples and the individual (age group, treatment), which enhances the accuracy and reliability of classification and regression techniques based on pattern recognition. It is noteworthy that our study, neither age groups nor antibiotic treatments were important indicators of AAD.

In conclusion, we are aware of the limitations of relatively small number of patients used in this study, the complex structure of the data, and the need for verification of our findings. The broad application spectrum of sequence-dependent fingerprinting techniques in the field of intestinal microbiology has been largely examined. It ranges from primary assessments of the bacterial complexity and diversity of intestinal community structures to the monitoring of compositional changes at different population levels upon dietary or therapeutic intervention (14) . In this model, causal modeling was based on current TTGE gel analysis and thus has the same limitations of any genetic analysis using biomolecular engineering (e.

g. DNA extraction, amplification). Therefore, it is possible that not all possible confounders are represented in the models, and some factors that are designated as no confounders might actually be so. Additional population-based studies with multivariable analyses structured on causal models are required to confirm the findings of this study. In addition, this study was primarily a hypothesis-generating study of resident microbiota genes, which utilized ever-improving molecular techniques and analyses, and demonstrates that important part of risk factors for AAD can be found within the individual microbiome. As such, it offers new perspectives for clinical applications at the level of prevention.

Acknowledgments:

We thank Dr Bertrand D. from INRA, Unité de Sensometrie-Chimiometrie (Nantes, France) for his assistance in this investigation.

Financial support: Biocodex, Inc.

Potential conflicts of interest: The authors declare no conflict of interest

References

1. **Arbuckle, R. B., S. L. Huber, and C. Zacker.** 2000. The consequences of diarrhea occurring during chemotherapy for colorectal cancer: a retrospective study. Oncologist **5:**250-9.
2. **Bartlett, J. G.** 2002. Clinical practice. Antibiotic-associated diarrhea. N Engl J Med **346:**334-9.
3. **Bartlett, J. G.** 2002. Clostridium difficile-associated Enteric Disease. Curr Infect Dis Rep **4:**477-483.
4. **Beaugerie, L., A. Flahault, F. Barbut, P. Atlan, V. Lalande, P. Cousin, M. Cadilhac, and J. C. Petit.** 2003. Antibiotic-associated diarrhoea and Clostridium difficile in the community. Aliment Pharmacol Ther **17:**905-12.
5. **Blakelock, R. T., and S. W. Beasley.** 2003. Infection and the gut. Semin Pediatr Surg **12:**265-74.
6. **Bosshard, P. P., S. Abels, R. Zbinden, E. C. Bottger, and M. Altwegg.** 2003. Ribosomal DNA sequencing for identification of aerobic gram-positive rods in the clinical laboratory (an 18-month evaluation). J Clin Microbiol **41:**4134-40.
7. **Collier, C. T., C. L. Hofacre, A. M. Payne, D. B. Anderson, P. Kaiser, R. I. Mackie, and H. R. Gaskins.** 2008. Coccidia-induced mucogenesis promotes the onset of necrotic enteritis by supporting Clostridium perfringens growth. Vet Immunol Immunopathol **122:**104-15.
8. **De La Cochetiere, M. F., T. Durand, V. Lalande, J. C. Petit, G. Potel, and L. Beaugerie.** 2008. Effect of Antibiotic Therapy on Human Fecal Microbiota and the Relation to the Development of Clostridium difficile. Microb Ecol.
9. **De La Cochetiere, M. F., H. Piloquet, C. des Robert, D. Darmaun, J. P. Galmiche, and J. C. Roze.** 2004. Early intestinal bacterial colonization and necrotizing enterocolitis in premature infants: the putative role of Clostridium. Pediatr Res **56:**366-70.
10. **Dethlefsen, L., S. Huse, M. L. Sogin, and D. A. Relman.** 2008. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol **6:**e280.
11. **Eckburg, P. B., E. M. Bik, C. N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S. R. Gill, K. E. Nelson, and D. A. Relman.** 2005. Diversity of the human intestinal microbial flora. Science **308:**1635-8.
12. **Hooper, L. V., M. H. Wong, A. Thelin, L. Hansson, P. G. Falk, and J. I. Gordon.** 2001. Molecular analysis of commensal host-microbial relationships in the intestine. Science **291:**881-4.
13. **Hrncir, T., R. Stepankova, H. Kozakova, T. Hudcovic, and H. Tlaskalova-Hogenova.** 2008. Gut microbiota and lipopolysaccharide content of the diet influence development of regulatory T cells: studies in germ-free mice. BMC Immunol **9:**65.
14. **Huys, G., T. Vanhoutte, and P. Vandamme.** 2008. Application of sequence-dependent electrophoresis fingerprinting in exploring biodiversity and population dynamics of human intestinal microbiota: what can be revealed? Interdiscip Perspect Infect Dis **2008:**597603.
15. **Iapichino, G., M. L. Callegari, S. Marzorati, M. Cigada, D. Corbella, S. Ferrari, and L. Morelli.** 2008. Impact of antibiotics on the gut microbiota of critically ill patients. J Med Microbiol **57:**1007-14.
16. **Janda, J. M., and S. L. Abbott.** 2007. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. J Clin Microbiol **45:**2761-4.
17. **Johnston, B. C., A. L. Supina, M. Ospina, and S. Vohra.** 2007. Probiotics for the prevention of pediatric antibiotic-associated diarrhea. Cochrane Database Syst Rev:CD004827.
18. **La Scola, B., V. A. Gundi, A. Khamis, and D. Raoult.** 2006. Sequencing of the rpoB gene and flanking spacers for molecular identification of Acinetobacter species. J Clin Microbiol **44:**827-32.

19. **Lepage, P., P. Seksik, M. Sutren, M. F. de la Cochetiere, R. Jian, P. Marteau, and J. Dore.** 2005. Biodiversity of the mucosa-associated microbiota is stable along the distal digestive tract in healthy individuals and patients with IBD. Inflamm Bowel Dis **11:**473-80.
20. **Manichanh, C., E. Varela, C. Martinez, M. Antolin, M. Llopis, J. Dore, J. Giralt, F. Guarner, and J. R. Malagelada.** 2008. The gut microbiota predispose to the pathophysiology of acute postradiotherapy diarrhea. Am J Gastroenterol **103:**1754-61.
21. **Martens, H., S. R. Veflingstad, E. Plahte, M. Martens, D. Bertrand, and S. W. Omholt.** 2009. The genotype-phenotype relationship in multicellular pattern-generating models - the neglected role of pattern descriptors. BMC Syst Biol **3:**87.
22. **McFarland, L. V.** 2008. Antibiotic-associated diarrhea: epidemiology, trends and treatment. Future Microbiol **3:**563-78.
23. **Seksik, P., P. Lepage, M. F. de la Cochetiere, A. Bourreille, M. Sutren, J. P. Galmiche, J. Dore, and P. Marteau.** 2005. Search for localized dysbiosis in Crohn's disease ulcerations by temporal temperature gradient gel electrophoresis of 16S rRNA. J Clin Microbiol **43:**4654-8.
24. **Simmon, K. E., A. C. Croft, and C. A. Petti.** 2006. Application of SmartGene IDNS software to partial 16S rRNA gene sequences for a diverse group of bacteria in a clinical laboratory. J Clin Microbiol **44:**4400-6.
25. **Simmon, K. E., S. Mirrett, L. B. Reller, and C. A. Petti.** 2008. Genotypic diversity of anaerobic isolates from bloodstream infections. J Clin Microbiol **46:**1596-601.
26. **Stringer, A. M., R. J. Gibson, J. M. Bowen, D. M. Keefe, R. M. Logan, A. S. Yeoh, and J. Hamilton.** 2009. Chemotherapy-induced modifications to gastrointestinal microflora: evidence and implications of change Gastrointestinal microflora and mucins may play a critical role in the development of 5-Fluorouracil-induced gastrointestinal mucositis. Curr Drug Metab **10:**79-83.
27. **Stringer, A. M., R. J. Gibson, J. M. Bowen, R. M. Logan, A. S. Yeoh, and D. M. Keefe.** 2007. Chemotherapy-induced mucositis: the role of gastrointestinal microflora and mucins in the luminal environment. J Support Oncol **5:**259-67.
28. **Suau, A., R. Bonnet, M. Sutren, J. J. Godon, G. R. Gibson, M. D. Collins, and J. Dore.** 1999. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl Environ Microbiol **65:**4799-807.
29. **Tannock, G. W.** 2001. Molecular assessment of intestinal microflora. Am J Clin Nutr **73:**410S-414S.
30. **Tannock, G. W.** 2007. What immunologists should know about bacterial communities of the human bowel. Semin Immunol **19:**94-105.
31. **Wistrom, J., S. R. Norrby, E. B. Myhre, S. Eriksson, G. Granstrom, L. Lagergren, G. Englund, C. E. Nord, and B. Svenungsson.** 2001. Frequency of antibiotic-associated diarrhoea in 2462 antibiotic-treated hospitalized patients: a prospective study. J Antimicrob Chemother **47:**43-50.
32. **Zoetendal, E. G., A. D. Akkermans, and W. M. De Vos.** 1998. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl Environ Microbiol **64:**3854-9.

Figure legends

Figure.1. Representative TTGE gel image of faecal microbiota DNA before antibiotic treatment. M control marker DNA.

Figure.2. Analysis of Variance (ANOVA). Fisher *F*-values (AAD) are plotted against the migration distance. The Fisher *F* limit (threshold value) was obtained using randomisation procedures (see Methods). Six electrophoresis distances were found to exceed this threshold, and were thus considered significant.

Figure.3. Mean TTGE electrophoresis at D_0. The grey bars emphasize the regions detected by ANOVA tests. Solid line, pattern from patients with AAD; Dotted line, pattern from patients without ADD.

Figure.4. Comparison of ANOVA and discriminant analysis. *Top :* regions detected by ANOVA (grey bars) *Bottom:* first six variables introduced in forward discriminant analysis (vertical lines). Vertical lines are numbered in order of introduction of the corresponding variable. Solid line, pattern from patients with AAD; Dotted line, pattern from patients without AAD.

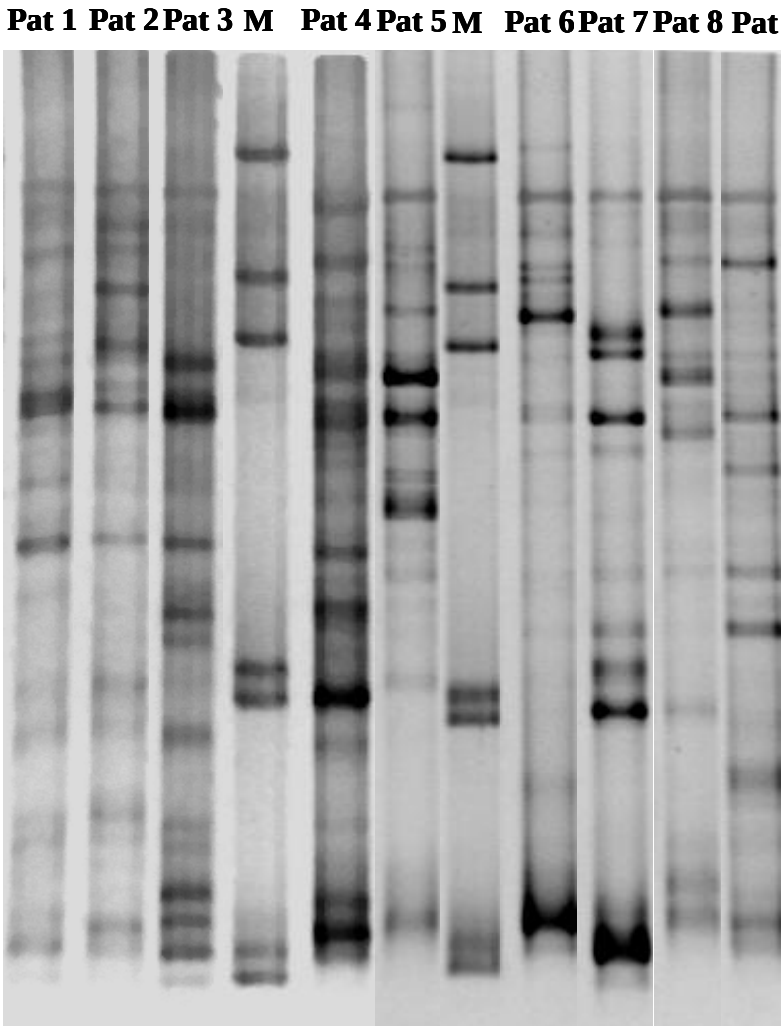


Figure 1

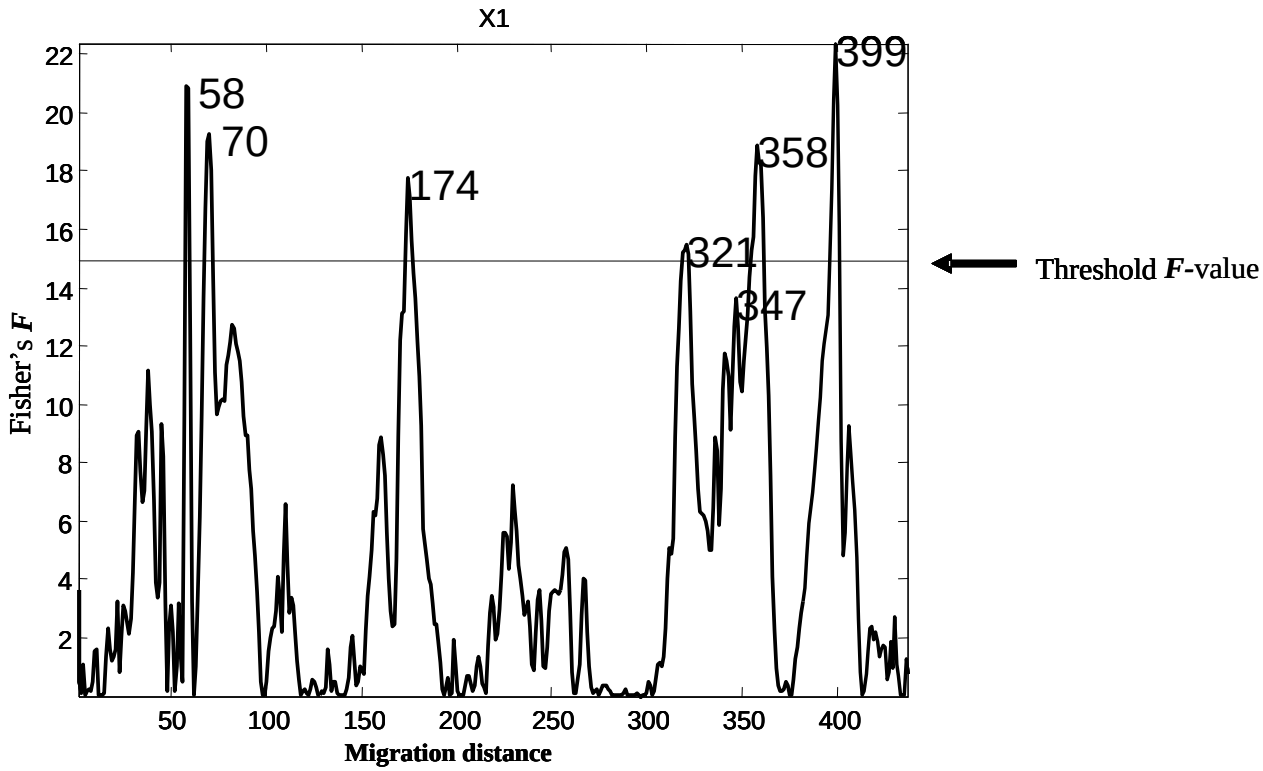

X1
58
70
174
321
347
358
399
Threshold *F*-value
Fisher's *F*
Migration distance
2
4
6
8
10
12
14
16
18
20
22
50
100
150
200
250
300
350
400

Figure 2

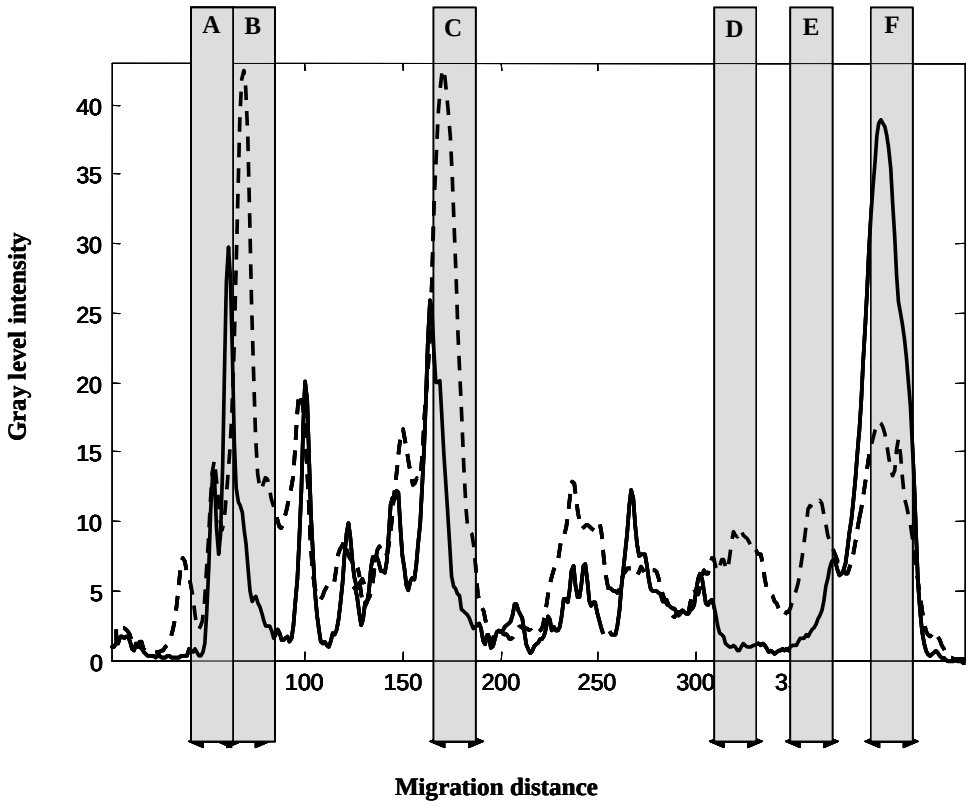

A
B
C
D
E
F
Gray level intensity
40
35
30
25
20
15
10
5
0
100
150
200
250
300
Migration distance

Figure 3

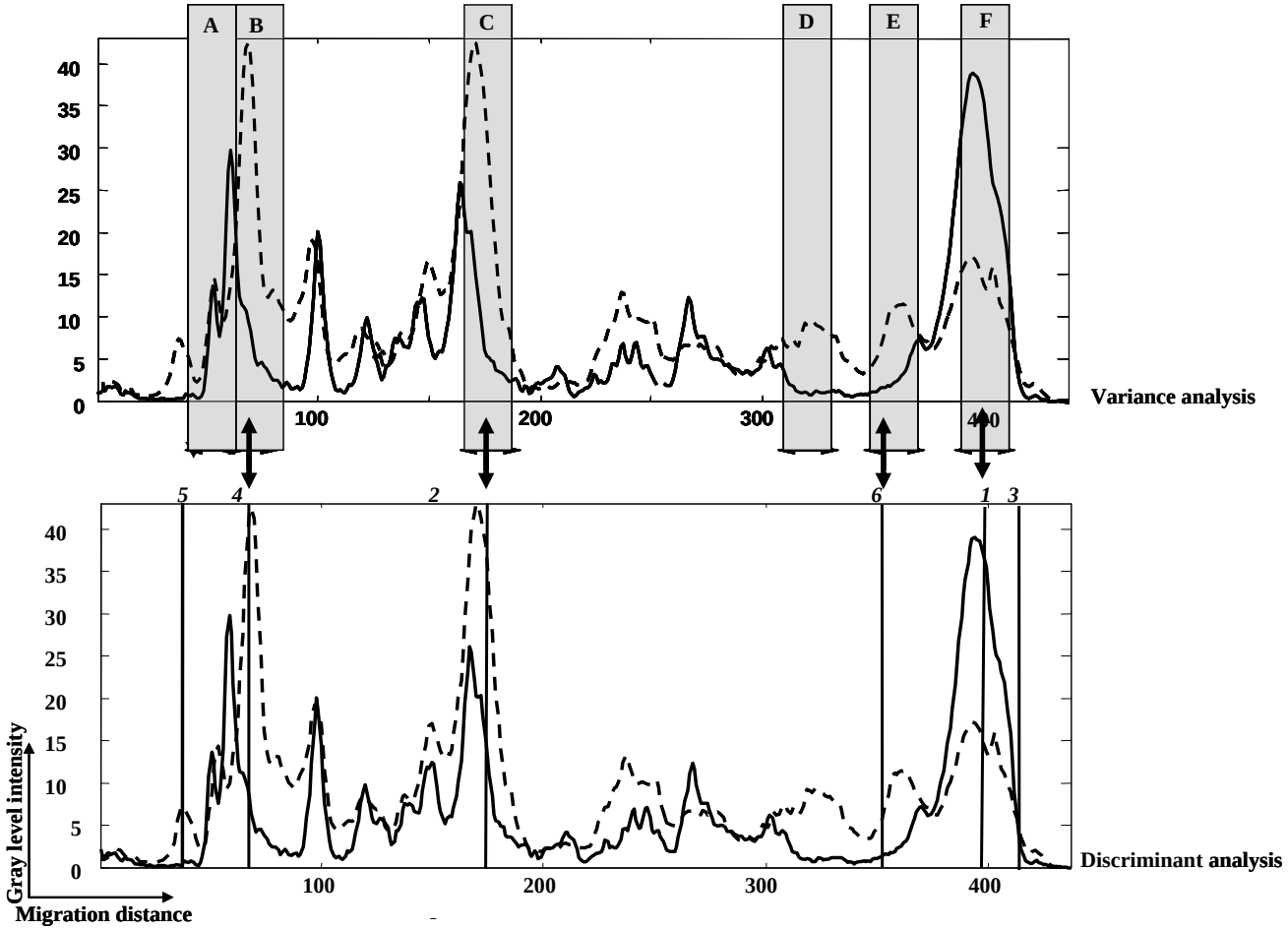
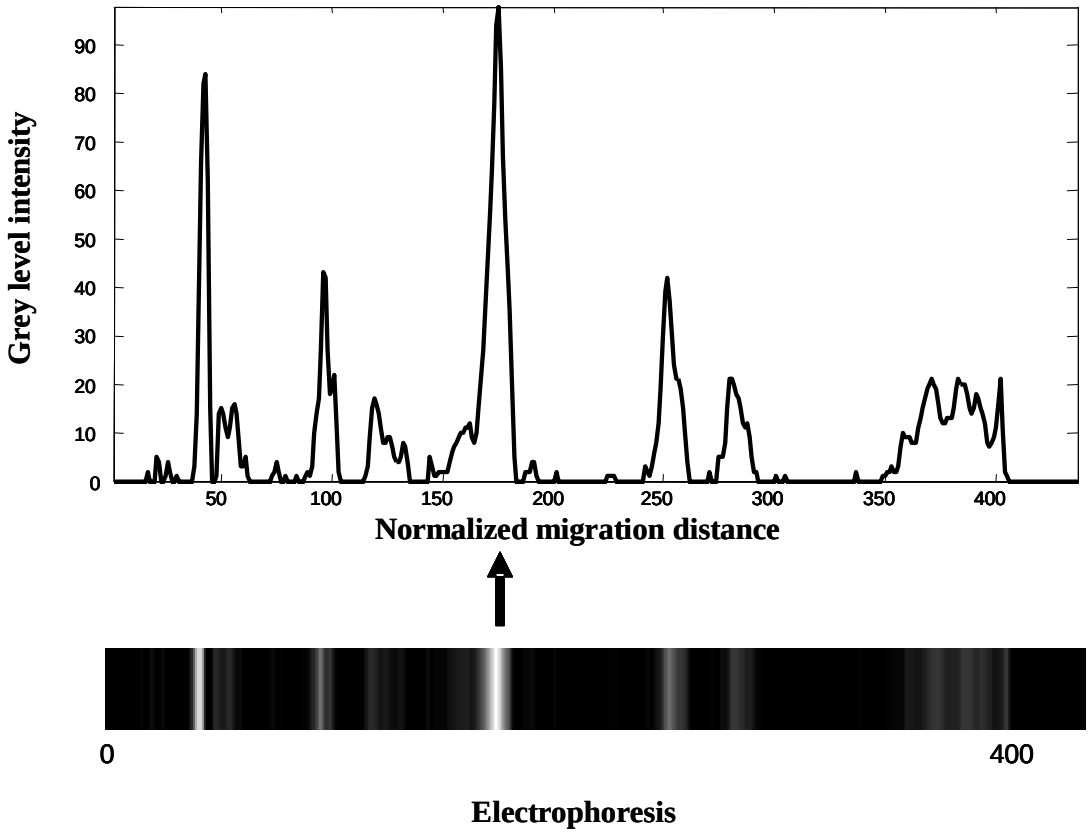

A
B
C
D
E
F
40
35
30
25
20
15
10
5
0
100
200
300
400
Variance analysis
5
4
2
6
1
3
Gray level intensity
Migration distance
Discriminant analysis

Figure 4



Complementary material