

Human Intestinal Microbiota Gene Risk Factors for Antibiotic-Associated Diarrhea: Perspectives for Prevention

Risk Factors for Antibiotic-Associated Diarrhea

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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 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.

Introduction

The collective effects of the intestinal microbiota are dictated by a complex network of interactions that span

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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 fecal microbiota and 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 by far the most common genetic marker used [16].

AAD is associated with altered intestinal microflora, mucosal integrity, vitamin and 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 2 months after the end of treatment [2, 17]. In critically ill patients, diarrhea increases morbimortality. 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 the diarrhea risk during antibiotic treatment is therefore worthwhile and may help control diarrhea in defined high-risk individuals; however, few data exist on risk factors for AAD.

The role of the human intestinal microbiota in health and specific diseases is a particularly important area of research [7, 12, 13]. Substantial progress has recently been made in characterizing the human intestinal microbiota, although its role in immune system development and regulation, nutrition, and pathogenesis of the host are still not well elucidated [30]. Furthermore, the rapid rate of microbial evolution, combined with the global rise of antimicrobial resistance and the low rate of novel antibiotic development underscores the urgent need for innovative therapeutics [5, 10]. Culture-based techniques have traditionally been used to determine the fecal microbiota. However, molecular techniques based on analysis of 16S rRNA genes directly amplified from bacterial DNA extracted from feces have estimated that

less than 25% of the fecal bacterial populations have been cultured to date [11, 28]. These approaches have provided considerable data about microbial ecosystems, including that of the human gastrointestinal tract. Detailed phylogenetic information has been obtained by cloning and sequencing 16S rRNA genes. Furthermore, several studies with fingerprinting of 16S rRNA genes have reported its benefits in monitoring community shifts [8, 19, 23, 29].

Hence, as a system model for the analysis of heterogeneous populations of bacteria, we analyzed the 16S rRNA genes in the genomes of all bacteria using temporal temperature gradient gel electrophoresis (TTGE) [6] and multivariable data-analysis [21]. In this retrospective study, we hypothesized that the susceptibility to diarrhea may be linked to the resident intestinal microbiota. For this purpose, we focused on a published clinical study [4], which included epidemiology forms, patient history, and laboratory reports; we analyzed database case records, specimen collection, and risk factors. We designed complex statistical analyses to determine the optimal procedures for providing the maximum relevant information with fingerprint data and obtaining knowledge about the 16S rRNA gene system.

Methods

Patients

Subjects enrolled in this study were adults (age range, 20–60 years) living in the Paris area. Subjects were prescribed a course of antimicrobial therapy for an ear, nose, or throat infection. Criteria for enrollment included prescription by a general practitioner for a 5-to-10-day course of antibiotics and age of 18 years or older. Potential candidates were excluded if they were institutionalized, had received antibiotic treatment during the previous 2 months, had been admitted to a hospital during the previous 6 months, had a known human immunodeficiency virus infection, had any allergy, or had experienced a bout of diarrhea (>2 loose stools per day) the day before enrollment. All patients provided informed written consent. Prescribed antibiotics were classified into one of the following groups: amoxicillin/clavulanic acid, other beta-lactam agents, or non-beta-lactam agents. Diarrhea was defined as the passage of at least three loose stools a day; AAD was defined as diarrhea associated with the administration of antibiotics (in the absence of any other obvious reason) during the 14-day study period. Each patient was asked to store the last stool before the beginning of the antibiotherapy (D_0) in a double-thickness container. The dominant microbiota profile at D_0 was considered to be patient's profile at equilibrium (i.e., the

resident microbiota). In addition to the molecular study, bacteriological investigations were performed according to standardized procedures.

DNA Isolation and 16S rRNA Gene Amplification

Immediately after fecal sample collection, total DNA was extracted from a 125-mg aliquot and purified as previously described [9]. DNA ($0.23 \pm 0.1 \mu\text{g}/\mu\text{l}$) was obtained from all samples. The DNA integrity and concentration (size, $> 21 \text{ kb}$) 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 step 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 gray 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 gray 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 years). 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 retention times predictive of the AAD: ANOVA which compares the mean values at each retention time 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 analyses was to detect the retention times where there were 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 with the value obtained with the following process: 10,000 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 D_0 stool samples (before antibiotherapy), 156 TTGE profiles were analyzed. The banding pattern was complex in all cases (Fig. 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

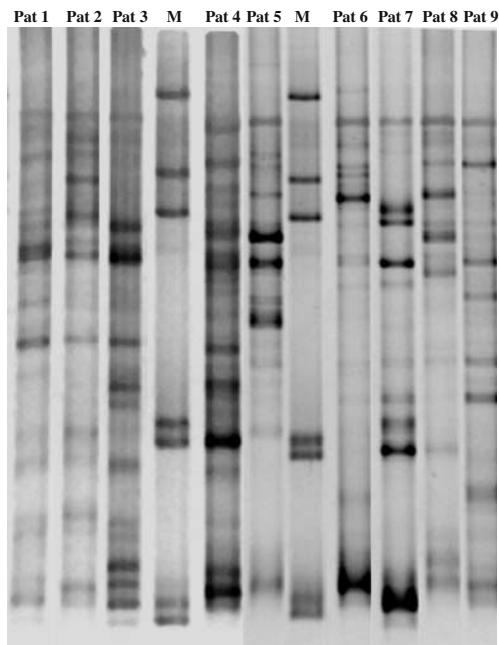


Figure 1 Representative TTGE gel image of fecal microbiota DNA before antibiotic treatment. *M* control marker DNA

bacterial 16S rRNA genes: each TTGE profile was converted into a curve, then digitized and further analyzed 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 that 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 gray 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 six variables allow clustering 142 among the 156 patients (91%) in the correct group.

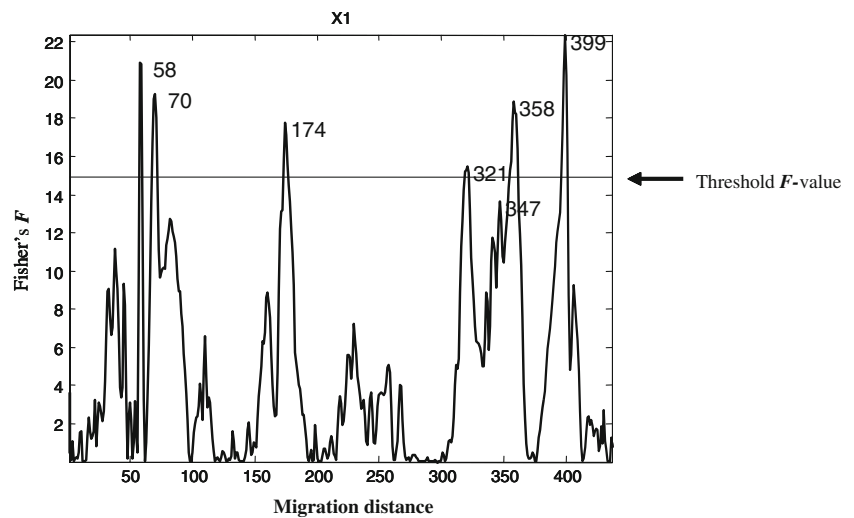
Individual Risk Prediction of AAD from Resident Intestinal Microbiota

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

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 randomization procedures (see **Methods**). Six electrophoresis distances were found to exceed this threshold and were thus considered significant



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

The important findings of this study are as follows:

1. Among the studied patient variables (antibiotherapy, age, sex, bacterial 16S rRNA genes), only the pre-antibiotic resident fecal bacterial 16S rRNA genes were found to be correlated to AAD.
2. 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.

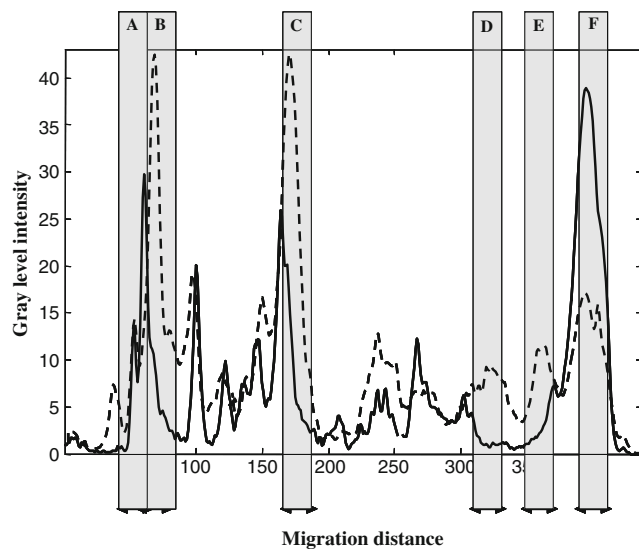


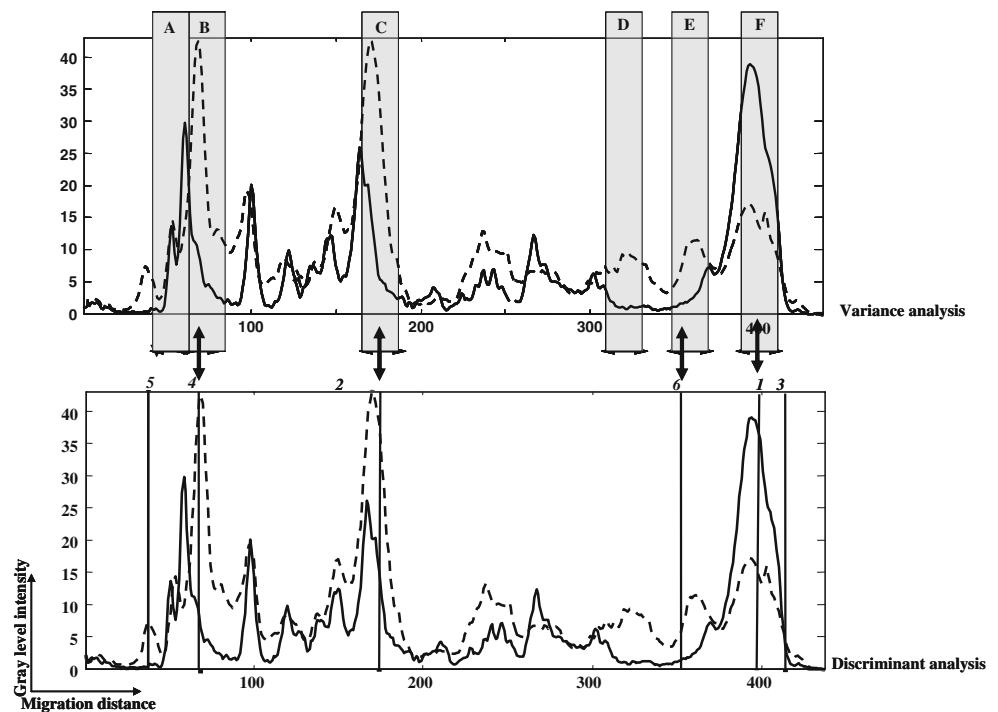
Figure 3 Mean TTGE electrophoresis at D_0 . The gray bars emphasize the regions detected by ANOVA tests. Solid line, pattern from patients with AAD; dotted line, pattern from patients without AAD

3. 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 fecal 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 fecal 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 a ~1,500-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 explained here, stand as one first step toward it. Therefore, the findings of this study

Figure 4 Comparison of ANOVA and discriminant analysis. *Top*: regions detected by ANOVA (gray 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



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 concluded 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). Furthermore, 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 the 16S rRNA genes. The next area of study will be to determine to which extent 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, in 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.

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

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