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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 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

63 **Introduction**

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

76 Antibiotic-associated diarrhea (AAD) is associated with altered intestinal microflora,
77 mucosal integrity, vitamin, mineral metabolism and crampy abdominal pain. If severe, AAD
78 may lead to electrolyte disturbances, dehydration, premature discontinuation of antibiotic
79 therapy, pseudomembranous colitis, toxic megacolon and possibly death. Reports in the
80 general population indicate that the incidence of AAD ranges from 25 to 62%, occurring at
81 any point from the initiation of therapy to two months after the end of treatment (2, 17). In
82 critically ill patients, diarrhea increases morbimortality. AAD leads also to longer hospital
83 stays and higher medical costs (1, 26, 27). The pathogenesis of AAD may be mediated
84 through the disruption of the normal microbiota and overgrowth of pathogens, or through
85 metabolic imbalances (3, 31). The individual risk for AAD varies greatly, influenced by host
86 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 (D₀) in a double-thickness container. The dominant microbiota profile at D₀
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 ($0.23 \pm 0.1 \mu\text{g}/\mu\text{l}$) was obtained from all samples. The DNA integrity
135 and concentration (size, >21kb) were determined by 1.5% agarose gel electrophoresis with

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

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

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

157 ***Data collection.*** The acquired data from all D₀ electrophoresis gels were collected as
158 147 retention times that resulted from digitizing each electrophoresis at equal distance
159 intervals. Each patient was also characterized by qualitative features related to age, sex,

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

162

163 *Multivariable data-analysis*

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

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

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

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

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

188

189

190 **Results**

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

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

203 **Multivariable data-analysis:** Using multivariate data analysis techniques to determine
204 potential causal factors for AAD, we found that only D₀ microbiota profiles were strongly
205 correlated with AAD. In this study, other factors (age, sex, and class of antibiotic) were not
206 relevant, thus we focused on the intestinal bacterial 16S rRNA genes: each TTGE profile was
207 converted into a curve, then digitized and further analysed to convert 16S rRNA genes to
208 numerical data (see complementary material). We applied advanced multivariate analysis to
209 confirm any predictive signature of the intestinal microbiota.

210 **Variance analysis.** Figure 2 shows the *F*- value of ANOVA carried out independently at each
211 data point of each electrophoresis at D₀. The studied factor was the presence/absence of AAD
212 after antibiotherapy. Six electrophoresis migration distance values were found exceed the
213 threshold *F*-value: (A) 58, (B) 70, (C) 174, (D) 321, (E) 358 and (F) 399. Figure 3 shows the
214 mean electrophoresis migration patterns associated with the absence and presence of AAD:
215 significant regions are emphasized using a grey color. Zones A and F are correlated to the
216 microbiota of patients with AAD, and zones B, C, D, and E are correlated to the microbiota of
217 patients without AAD.

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

221
222 **Individual risk prediction of AAD from resident intestinal microbiota.** Figure 4
223 demonstrates the degree of agreement between ANOVA and FLDA. Four of the first six
224 variables selected by the discriminant analysis were also identified by ANOVA, producing
225 distinct profiles for AAD and no AAD. The risk prediction for any new case of AAD could
226 be calculated from its intestinal microbiota gene fingerprinting with an error of 2% in the case
227 of AAD (1/44) and 11% (12/112) in the case of no AAD.

228

229 **Discussion**

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

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

237

238 The important findings of this study are as follows:

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

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

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

248

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

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

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

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

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

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

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

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

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

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

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452 **Figure legends**

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

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

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

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

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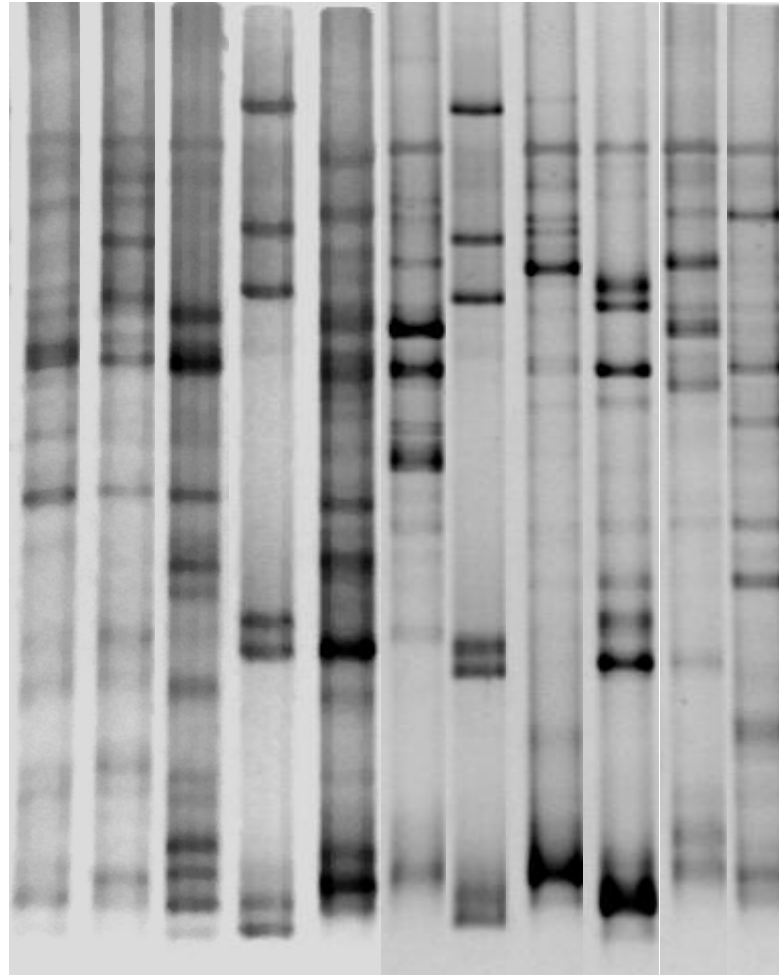


Figure 1

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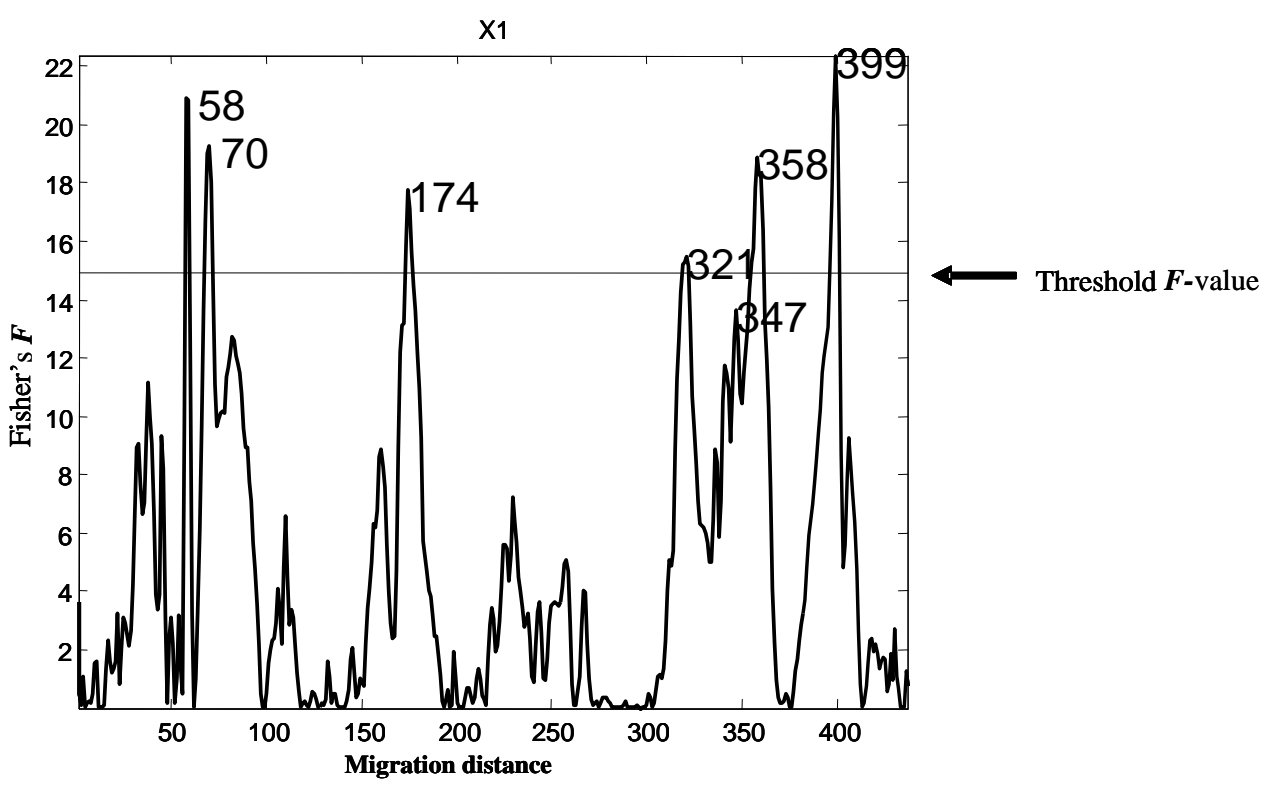


Figure 2

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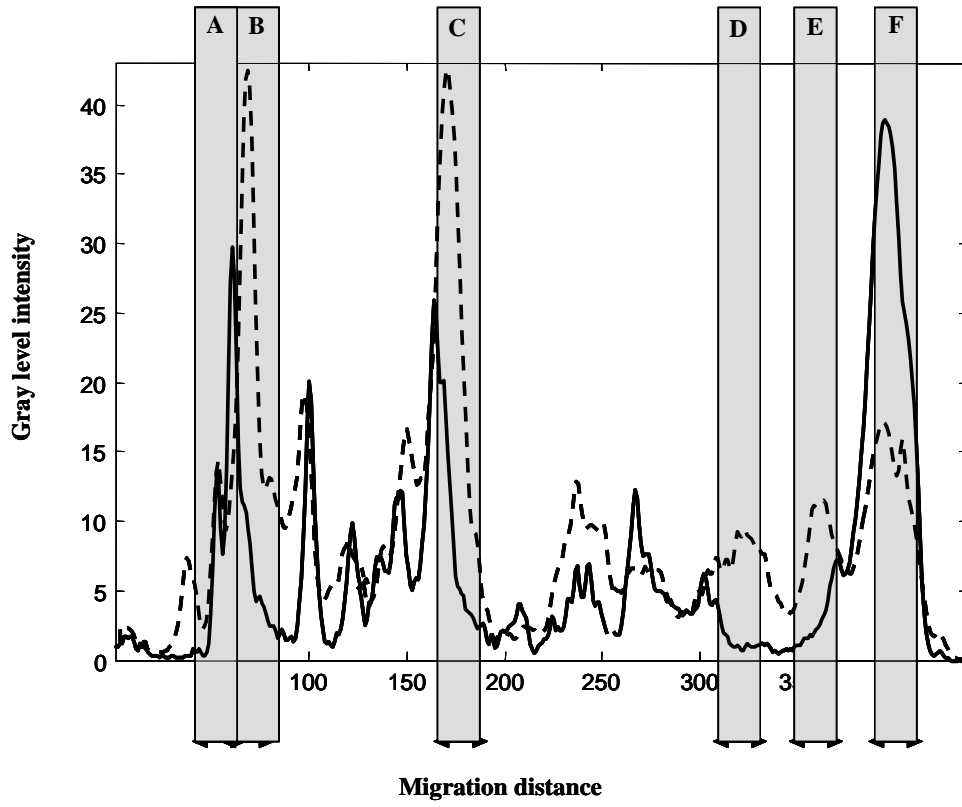


Figure 3

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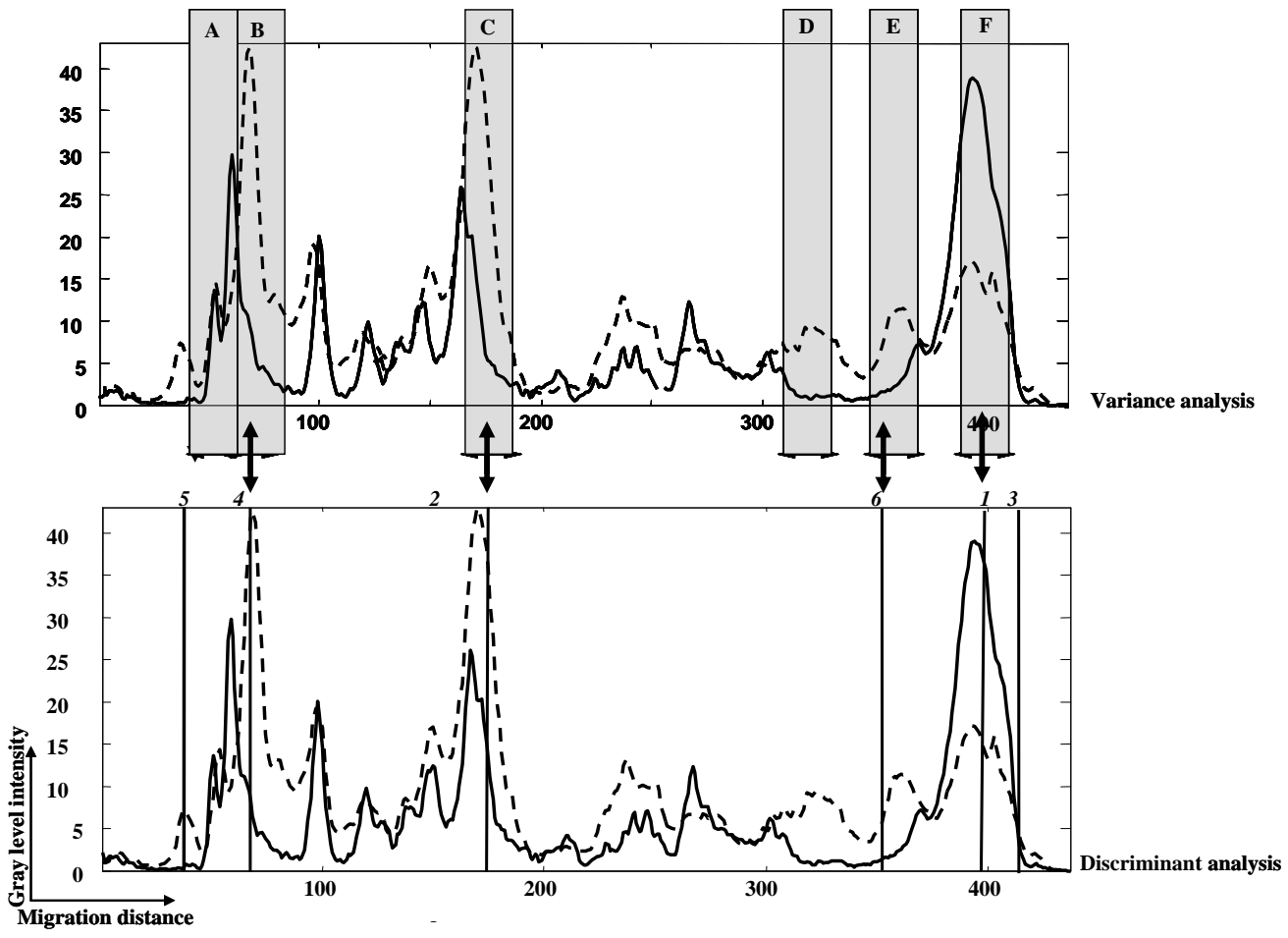


Figure 4

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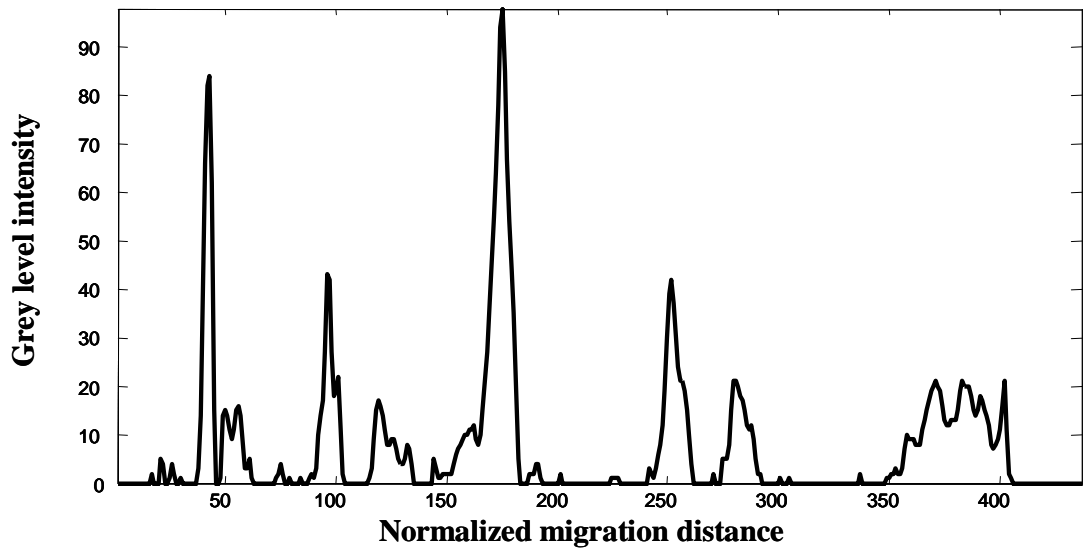
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Electrophoresis

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638 **Complementary material**

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