

# Characteristics of human intestinal *Escherichia coli* with changing environments

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## Summary

To investigate if the characteristics of human intestinal *Escherichia coli* are changing with the environment of the host, we studied intestinal *E. coli* from subjects having recently migrated from a temperate to a tropical area. We determined the phylogenetic group, the prevalence of the antibiotic resistance, the presence of integrons and the strain diversity in faecal isolates from 25 subjects originally from metropolitan France and expatriated to French Guyana. These characteristics were compared with those of 25 previously studied Wayampi Amerindian natives of French Guyana and from 25 metropolitan French residents. The three groups of subjects were matched for age and sex, had not taken antibiotics for at least 1 month, nor had been hospitalized within the past year. In all, the characteristics of intestinal *E. coli* from Expatriates were intermediate between those found in residents from metropolitan France and those found in

natives of French Guyana. Prevalence of carriage of resistant Gram-negative bacteria in Expatriates was intermediate between French residents and Wayampi as were the prevalence of integrons in *E. coli* (12.3% versus 16.3% and 7.8% respectively), and the intra-host diversity of *E. coli* (2.3 strains/subject versus 1.9 and 3.1, respectively); lastly, in Expatriates, the prevalence of carriage of phylogenetic group B2 strains was lower than in French residents (16% versus 56%,  $P = 0.005$ ), while carriage of phylogenetic group A strains was lower than in Wayampi (56% versus 88%,  $P = 0.03$ ). Our results suggest that the composition of the commensal intestinal flora of humans is not static but changes dynamically in response to new environmental conditions.

## Introduction

*Escherichia coli*, a commensal of the intestinal tract of healthy humans can also cause a wide array of diseases, from diarrhoea to extraintestinal infections (Kaper *et al.*, 2004), and be multidrug resistant, which impacts public health and clinical practice (20). Resistance results most often from horizontal gene transfer, mediated by plasmids and transposons carrying integrons able to capture, integrate and express antibiotic-resistance genes (Mazel, 2006). *Escherichia coli* has a clonal genetic population structure made of four phylogenetic groups, A, B1, B2 and D (Herzer *et al.*, 1990), with a rate of genetic recombination between these groups which is low for some authors (Lecointre *et al.*, 1998) and high for others (Wirth *et al.*, 2006).

Major genomic differences have been described between isolates of these four phylogenetic groups by results of multilocus enzyme electrophoresis (Selander and Levin, 1980) then by those of multilocus sequence typing (Wirth *et al.*, 2006). A rapid and simple PCR method has been proposed to assign isolates to one of these four phylogenetic groups (Clermont *et al.*, 2000). Extraintestinal isolates belong mainly to the B2 phylogenetic group, and at a lesser extent to the D phylogenetic group (Bingen *et al.*, 1998; Johnson *et al.*, 2005). Both groups have a higher prevalence of extraintestinal virulence determinants than do strains in A and B1 phylogenetic groups (Picard *et al.*, 1999). Strains from the phylogenetic group B2 remain over time, more susceptible

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than other phylogenetic groups to antibiotics (Johnson *et al.*, 1991), through a complex and not fully understood relationship (Kuntaman *et al.*, 2005; Moreno *et al.*, 2006). Pathogenic strains associated with severe and acute diarrhoea are distributed outside the B2 group (Escobar-Páramo *et al.*, 2003; Wirth *et al.*, 2006). In commensal flora, we found significantly less B2 strains in subjects from tropical areas and less group A strains from humans residing in temperate zones, suggesting a relationship between environment and the phylogenetic group of *E. coli* that colonizes the intestine, a relationship also found by others (Duriez *et al.*, 2001; Escobar-Paramo *et al.*, 2004; Pallecchi *et al.*, 2007). Strains isolated from subjects living in tropical areas also appeared more genetically diverse (Escobar-Paramo *et al.*, 2004). In addition, studying commensal *E. coli* strains from pig farmers (Aubry-Damon *et al.*, 2004) or Wayampi Amerindians (Grenet *et al.*, 2004; Skurnik *et al.*, 2005) who had not had direct recent exposure to antimicrobials nor been hospitalized for at least 1 year, we nonetheless found high rates of antibiotic resistance. We thus hypothesized that beside direct antibiotic exposure, high resistance rates in intestinal enterobacteria, as well as integron prevalence

in commensal *E. coli*, were influenced by specific, non-antibiotic, environmental selective pressures leading to the spread of the more resistant strains.

To further explore the complex relationship between intestinal *E. coli* carriage and the environment of the host, we compared the prevalence of antibiotic resistance and integrons, as well as the phylogenetic structure and strain diversity, among *E. coli* isolates from the faeces of carefully matched subjects that differed in their living environments: French residents, French expatriates in Guyana and local Wayampi Amerindians in Guyana. Our results suggest that as humans move to different areas of the world the faecal *E. coli* flora changes progressively to become more like that found in longer-term residents of a local environment.

## Results

### *Characteristics of the predominant E. coli isolates from different areas (Table 1)*

Except for a significantly higher prevalence of resistance to tetracycline in the *E. coli* isolated from the Wayampi, no significant difference in antibiotic resistance rates

**Table 1.** Prevalence and comparison between the three populations studied of characteristics of the *E. coli* from their dominant faecal flora and of the enterobacteria from their subdominant faecal flora.

| Characteristics                           | Parameters      | Populations                       |                              |                          | P-values                          |   |                               |
|---|-----------------|-----------------------------------|------------------------------|--------------------------|-----------------------------------|---|-------------------------------|
|   |                 | French residents<br><i>n</i> = 25 | Expatriates<br><i>n</i> = 25 | Wayampi<br><i>n</i> = 25 | French resident<br>versus Wayampi | Expatriates<br>versus French<br>residents | Expatriates<br>versus Wayampi |
| <b>Predominant flora (<i>E. coli</i>)</b> |                 |                                   |                              |                          |                                   |   |                               |
| Resistance                                | Ampicillin      | 22%                               | 22%                          | 23%                      | NS                                | NS  | NS                            |
|   | Streptomycin    | 38%                               | 22%                          | 29%                      | NS                                | NS  | NS                            |
|   | Kanamycin       | 0%                                | 0                            | 2%                       | NS                                | NS  | NS                            |
|   | Tetracycline    | 32%                               | 31%                          | 50%                      | 0.02                              | NS  | 0.02                          |
|   | Chloramphenicol | 20%                               | 7%                           | 14%                      | NS                                | NS  | NS                            |
|   | Gentamicine     | 0                                 | 0                            | 0                        | NS                                | NS  | NS                            |
|   | Ceftazidime     | 0                                 | 0                            | 0                        | NS                                | NS  | NS                            |
|   | Nalidixic acid  | 0                                 | 0                            | 0                        | NS                                | NS  | NS                            |
|   | Pefloxacin      | 0                                 | 0                            | 0                        | NS                                | NS  | NS                            |
| Cotrimoxazole                             | 10%             | 12%                               | 16%                          | NS                       | NS                                | NS  |                               |
| Phylogenetic group                        | A               | 51%                               | 56%                          | 88%                      | 0.02                              | 0.03                                      | 0.03                          |
|   | B1              | 24%                               | 24%                          | 72%                      | < 0.01                            | NS  | 0.005                         |
|   | D               | 32%                               | 76%                          | 40%                      | NS                                | 0.005                                     | 0.02                          |
|   | B2              | 56%                               | 16%                          | 8%                       | NS                                | 0.005                                     | 0.005                         |
| Diversity                                 |                 | 1.9                               | 2.3                          | 3.1                      | 0.03                              | NS  | NS                            |
| Integrons                                 |                 | 16.3%                             | 12.3%                        | 7.8%                     | NS                                | NS  | NS                            |
| <b>Subdominant flora (enterobacteria)</b> |                 |                                   |                              |                          |                                   |   |                               |
| Carriage of resistance                    | Ampicillin      | 48%                               | 88%                          | 100%                     | < 0.001                           | 0.002                                     | 0.08                          |
|   | Streptomycin    | 44%                               | 76%                          | 100%                     | < 0.001                           | 0.005                                     | 0.01                          |
|   | Kanamycin       | 24%                               | 48%                          | 68%                      | 0.004                             | 0.06                                      | NS                            |
|   | Tetracycline    | 40%                               | 68%                          | 100%                     | 0.004                             | 0.02                                      | 0.005                         |
|   | Chloramphenicol | 12%                               | 36%                          | 68%                      | 0.001                             | 0.02                                      | 0.03                          |
|   | Gentamicine     | 0%                                | 28%                          | 32%                      | 0.003                             | 0.08                                      | NS                            |
|   | Ceftazidime     | 0%                                | 4%                           | 16%                      | NS                                | NS  | NS                            |
|   | Nalidixic acid  | 8%                                | 0%                           | 12%                      | NS                                | NS  | NS                            |

was found between the three populations, whatever the marker considered.

Concerning the distribution of phylogenetic groups we observed that, in Expatriates, the prevalence of carriage of the phylogenetic groups was (i) significantly lower than in French residents for the phylogenetic group B2 (16% versus 56%,  $P < 0.005$ ), (ii) significantly lower than in Wayampi for the phylogenetic B1 (24% versus 72%,  $P < 0.01$ ), and (iii) significantly higher than in French residents and Wayampi for the phylogenetic group D (76% versus 32%,  $P < 0.01$ ) and (76% versus 40%,  $P < 0.05$ ) respectively. The prevalence of carriage of the phylogenetic group A was lower in French residents than in Wayampi (51% versus 88%,  $P < 0.05$ ). No significant difference in carriage of the phylogenetic group A was found between French residents and Expatriates (51% versus 56%). The intra-host diversity of *E. coli* was significantly higher in the Wayampi (3.1 strains/subject) than in French residents (1.9 strains/subject) ( $P < 0.05$ ). In Expatriates, it was intermediate between Wayampi and French residents (2.3 strains/subject).

The prevalence of integrons in Expatriates isolates was of 12.3% (7/57) being intermediate between those found in isolates from French residents and Wayampi (16.3% and 7.8% respectively) (18). We found six class 1 integrons and one class 2 integron but no class 3. Class 1 integrons were more prevalent in isolates resistant to any one of the antibiotics tested, than in the susceptible strains (data not shown). Integron prevalences were 17.7% (3/17) and 13.8% (4/29) in isolates from phylogenetic groups A and D respectively. No integrons were found in isolates from the phylogenetic groups B2 ( $n = 5$ ) nor B1 ( $n = 6$ ). Four of the class 1 integrons had one gene cassette (*aadA1*) and two had two (*aadA1-dfrA1*). The single class 2 integron had four gene cassettes (*dfr-aad-sat-orfX*).

#### *Antibiotic resistance in the subdominant flora (Table 1)*

The prevalence of antibiotic resistance for ampicillin, streptomycin, kanamycin, tetracycline, chloramphenicol and gentamicin among members of the family *Enterobacteriaceae* in the subdominant flora was significantly lower in French residents with no exposure to the Guyanese environment than in the Wayampi. In the Expatriates with intermediate exposure, the prevalence of resistance for these markers was also intermediate, the differences reaching statistical significance with either of the two comparative groups in 13 out of 18 instances or being close in three additional ones (0.08 for ampicillin and gentamicin and 0.06 for kanamycin).

#### **Discussion**

We found that several characteristics of commensal enterobacteria of French expatriates in French Guyana

staying there for 6–24 months were different from those of residents in metropolitan France and were becoming more similar to those of native Wayampi Amerindians living permanently in the territory. Because the subjects from each population were carefully matched for age and sex and had not taken antibiotics for at least 2 months, this suggests that environment was influencing the composition in the intestinal flora which was changing in the Expatriates during the time of exposure in a new environment.

In the predominant intestinal enterobacteria, several genetic characteristics were intermediate or becoming the same in expression level when comparing *E. coli* strains isolated from Expatriates with French residents and Wayampi. The prevalence of phylogenetic group B2 was significantly lower in Expatriates and Wayampi than in French residents, but not different between the Expatriates and the Wayampi (Table 1). This is consistent with a previous observation that subjects living in tropical areas carry fewer strains of group B2 than those living in temperate zones (Escobar-Paramo *et al.*, 2004). In this previous work, it has been suggested that as tropical populations seem to preferentially harbour strains of group A and to a lesser extent B1, these strains might have the genetic background necessary for the emergence of pathogenic intestinal strains. This might be one of the factors explaining the higher incidence of diarrhoea in tropical countries. However, not all the phylogenetic groups were changing in the Expatriates, as if we found fewer strains from the phylogenetic groups A and B1 in this group than in Wayampi, the rates of isolation were identical between Expatriates and French residents (Table 1). This could suggest that strains in phylogenetic group A and B1, by contrast to those of phylogenetic groups B2 and D, might be maintained in an individual's gastrointestinal tract. The difference of the prevalence of B2 isolates between French residents, Expatriates and Wayampi are most probably not only due to the climatic changes but also to the change of the local environment that may influence the gut flora. The diet, particularly, is known to play a major role (Gordon and Cowling, 2003; Pupo *et al.*, 2000; Escobar-Paramo *et al.*, 2006). Also, the level of hygiene could increase the turnover rate of *E. coli* isolates in an individual's gastrointestinal tract as it has been observed in Pakistani infants in comparison with those to Swedish infants (Adlerberth *et al.*, 1998). Finally, diet modifications have also been suggested as a key factor determining the decrease of the prevalence of the B2 isolates along the time in French student living in the same geographic site (Escobar-Paramo *et al.*, 2004). Even if we had no information on the diet of the Expatriates and French residents, we can suppose that it was modified between these two groups.

The prevalence stability of A and B1 isolates between Expatriates and French residents might be based more on

microbial or host genetic background than on diet modification, host hygiene and geographical environment. The higher prevalence of strains from phylogenetic group D in Expatriates than in French residents and Wayampis is unexpected. It is, in our knowledge, the highest prevalence of strains from phylogenetic group D never observed in commensal flora. The reasons of this modification are unclear and further studies such in animal model are required to clarify it.

Also, there was more diversity among *E. coli* isolates from the predominant flora of the Wayampi than among isolates from French residents, while the diversity was intermediate in *E. coli* from Expatriates (Table 1). This is in agreement with the previously described impact of climatic/geographic factors on the diversity of commensal *E. coli* (Escobar-Paramo *et al.*, 2004).

In Expatriates, the prevalence of integrons was intermediate between those in French residents and in the Wayampi. Also, the presence of integrons was significantly associated with antibiotic resistance and there were no integrons in the phylogenetic group B2 strains. This confirmed previous results suggesting that the prevalence of integrons in the microbiota depended not only on antibiotic exposure but also on which phylogenetic groups are found in a particular environment (Skurnik *et al.*, 2005). The low diversity of the gene cassettes found was also in agreement with previous works (Skurnik *et al.*, 2005; Skurnik *et al.*, 2006).

In the Gram-negative bacteria from the subdominant faecal flora of the Expatriates, the prevalence of carriage of commensal-resistant enterobacteria was higher than in the French residents but lower than in the Wayampi. As these differences on the antibiotic resistance were only observed in the subdominant enterobacteria, and not in the predominant *E. coli*, we confirmed that the subdominant flora may be a more sensitive indicator of differences in antibiotic resistance in intestinal commensal bacteria when comparing rates among population groups (Lester *et al.*, 1990; Aubry-Damon *et al.*, 2004).

Considering all these results, one could argue that the differences observed between Expatriates and the other populations are due, in part, to differences in lifestyle and hygiene. However, these factors did not differ much between the Expatriates and the French residents. Thus, the significant differences observed between these two populations could better be ascribed to the consequences of living in different environments. We believe this is best explained by the Expatriates living in an environment close to that of the native Guyanese but with a way of life closer to that of residents living in metropolitan France.

Our study had some limitations. First, we did not sample the same subjects before and after they migrated. We did not do so because such a study is difficult to perform and a high number of subjects are lost to follow up. Therefore,

we cannot fully exclude the possibility that genetic differences between Wayampi and subjects from metropolitan France might account for some of the differences observed for *E. coli* colonization. Additionally, the three groups were not sampled concomitantly. However, the time elapsed between the sampling periods was only 3 years, which is short when compared with the pace of change in antibiotic resistance, and this should not have significantly impacted the results.

In conclusion, and in spite of these limitations, our results suggest that environment influences the composition of the commensal intestinal flora of humans.

## Experimental procedures

### Populations

We compared commensal *E. coli* from three adult populations with no, intermediate or full exposure to a tropical environment. Individuals with no exposure were bank and insurance workers from metropolitan France, those with intermediate exposure were French military police members or families expatriated to Cayenne, the capital city of French Guyana, who had arrived from metropolitan France between 6 and 24 months before the study, and individuals with full exposure were Wayampi Amerindians living permanently in the southern part of French Guyana. The samples from the volunteers were obtained in 2001, 2003 and 2000 respectively. All volunteers were healthy and had in common no direct exposure to antibiotics for at least 2 months nor any record of hospitalization for at least 1 year.

Mode of recruitment, sampling and harvesting of faeces, as well as part of the data on resistance in commensal *E. coli* from the French residents (Aubry-Damon *et al.*, 2004) and the Wayampi Amerindians (Grenet *et al.*, 2004) were published previously. The expatriated military police members and families were specifically recruited and sampled for the purpose of the present study, using methods identical to those used in the two other studies. Briefly, in these Expatriates potential volunteers were approached by the physician in charge of their health care and asked to take part in the study and sign an informed consent form. They were then clinically examined and asked to bring a sample of freshly passed faeces (< 12 h). Exclusion criteria were physical illness at the time of the study, including fever or any other infectious symptom, sore throat or unformed stools at the time of sampling and antibiotic ingestion during the preceding 2 months. This last criterion was retrospectively verified on the computerized reimbursement data of the French social security system. Sample harvesting, strain isolation and storage followed the same procedure as for residents of metropolitan France and Wayampi Amerindians.

Subjects in each of the three populations were matched for sex and age (within 5 years). When several subjects from one population met the matching criteria, only one of them, chosen at random, was included in the group. The final sample studied was made of 25 subjects from each group (13 men and 12 women aged 20–65 years).

Data on the prevalence of antibiotic resistance in the dominant flora and in the subdominant intestinal enterobacteria

and data on integron content, phylogenetic grouping and strain diversity of the *E. coli* of these subjects were gathered either from the database of our previously published studies (Aubry-Damon *et al.*, 2004; Grenet *et al.*, 2004; Skurnik *et al.*, 2005) or via additional experiments using the same techniques as used in these published reports.

### Microbiology

**Characteristics of the predominant *E. coli*.** Five lactose positive colonies were randomly selected after culture of faecal samples on Drigalski agar without antibiotics and *E. coli* identification was confirmed using API 20E strips (API, La Balmes-Grottes, France). Antibiotic susceptibilities of each isolate to ampicillin, gentamicin, ceftazidime, streptomycin, kanamycin, nalidixic acid, tetracycline, chloramphenicol, pefloxacin and cotrimoxazol was determined by the disk diffusion method, as recommended by the Antibiogram Committee of the French Society for Microbiology (<http://www.sfm.asso.fr>). A volunteer was defined as a carrier of *E. coli* resistant to a given antibiotic in the predominant flora when at least one of these isolates was resistant to this antibiotic.

Presence of class 1, 2 and 3 integrons was determined using a Magna Pure LC apparatus (Roche, Mannheim, Germany) according to the manufacturer's recommendations using triplex real-time PCR on an ABI Prism 7000 SDS thermocycler (Applied BioSystems, Courtaboeuf, France) using specific primer pairs, as described (Skurnik *et al.*, 2005). Gene cassettes were characterized in the integron positive strains, as described (Skurnik *et al.*, 2005).

Phylogenetic grouping of isolates was determined using a triplex PCR based on the presence or absence of three DNA fragments (*chuA*, *yjaA* and TspE4C2), as described (Clermont *et al.*, 2000). The combination of presence/absence of the three DNA fragments allows the delineation of phylogenetic groups A, B1, B2 and D. When several isolates from the same subject displayed identical antibiotic susceptibility patterns and belonged to the same phylogenetic group, they were considered as replicates (Skurnik *et al.*, 2005), and only one was selected at random for further analysis. The diversity of *E. coli* in each group was defined as the mean number of different isolates included in the analysis, per subject.

**Antibiotic resistance in the subdominant intestinal enterobacteria.** In addition to the study of the resistance of the *E. coli* from the predominant intestinal enterobacteria, the prevalence of resistance was also assessed in any members of the family *Enterobacteriaceae* present in the subdominant flora, because it has been suggested that this could be a more sensitive means to detect carriage of antibiotic resistant bacteria than evaluation of the predominant *E. coli* clones (Aubry-Damon *et al.*, 2004; Lester *et al.*, 1990). Faecal suspensions were plated onto Drigalski agar containing concentrations of antibiotics, chosen as described (<http://www.sfm.asso.fr>) to differentiate susceptible from susceptible isolates either ampicillin, gentamicin, ceftazidime, streptomycin, kanamycin, nalidixic acid, tetracycline or chloramphenicol. A volunteer was defined as a carrier of enterobacteria resistant to a given antibiotic in the subdominant flora when at least one isolate, confirmed

as resistant by the disk diffusion method, was recovered from the plate containing it.

### Statistical analysis

The chi-square test was used for between-group comparisons, or the Fisher exact test when the expected frequencies were under 5. The MacNemar's test was used for between-group comparisons of matched observations. A *P*-value of < 0.05 was considered statistically significant. The Shapiro–Wilk test was used to assess the normality of the *E. coli* diversity distribution in each group of subjects, and the Bartlett's test was used to test the equality of the diversity variances between the groups. Thus, when these two hypotheses were verified, a one-way analysis of variance was performed to compare the diversity between the three groups. When the hypotheses of normality and equality of variances were not verified, the Kruskal–Wallis rank test was used. Quantitative variables were summarized by means and 95% confidence intervals, and qualitative ones by frequencies and percentages.

For multiple between-group comparisons, the Bonferroni's correction was used to assess significant differences at a global 5% level. Statistical analyses were performed with Stata software, version 8.0 (Stata).

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