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Effect of Amoxicillin-Clavulanic Acid on Human Fecal Flora in a Gnotobiotic Mouse Model Assessed with Fluorescence Hybridization Using Group-Specific 16S rRNA Probes in Combination with Flow Cytometry

Marie Claude Barc,[1] François Bourlioux,[1] Lionel Rigottier-Gois,[2] Céline Charrin-Sarnel,[1] Claire Janoir,[1] Hélène Boureau,[1] Joël Doré,[2] and Anne Collignon[1]*

Unité associée INRA "Ecosystème Microbien Digestif et Santé," Faculté de Pharmacie, Université Paris-Sud, 92296 Châtenay-Malabry Cedex,[1] and Unité d'Ecologie et de Physiologie du Système Digestif, Institut National de la Recherche Agronomique, Domaine de Vilvert, 78852 Jouy en Josas Cedex,[2] France

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Predominant groups of bacteria from a human fecal flora-associated mouse model challenged with amoxicillin-clavulanic acid were quantified with fluorescence in situ hybridization combined with flow cytometry using specific 16S rRNA targeted oligonucleotide probes. This approach provides a useful tool with high throughput to evaluate fecal microflora under antibiotic treatment.

The intestinal tract of humans is a complex bacterial ecosystem, and the composition of the normal microflora plays an important role in human health and disease with regard to nutrition and the immunological balance of the host (6, 20). This microflora is subjected to variations according to diet, environment, and treatments. Antibiotic treatments, particularly, can disrupt the balance of the flora and the barrier effect.

Studies concerning the effect of amoxicillin-clavulanic acid on intestinal flora in human volunteers or animal models have been carried out with traditional culture. About 50% of intestinal species are as yet unknown due to the complexity of the digestive ecosystem, consisting mainly of obligatory anaerobic bacteria, which are difficult to cultivate or uncultivable. Recent advances in the analysis of such complex microbial ecosystems have involved molecular analysis based on hybridization with group-specific 16S rRNA oligonucleotide probes. Dot blot hybridization with radiolabeled probes has been assessed for characterization of fecal microflora (3, 15). Fluorescence in situ hybridization (FISH) has been developed (23) in combination with either microscopic (2, 7) or flow cytometry detection (1, 18, 25). These techniques have provided specific identification, and uncultivable bacteria have been identified. Studies have been carried out to measure the stability of the microflora in a gnotoxenic mouse model associated with an intestinal human microflora (17, 27). This animal model is convenient for studying modifications of the intestinal ecosystem and makes screening before clinical trials possible.

This study aims at comparing the composition of the fecal flora in a human flora-associated mouse model during and after a 7-day oral treatment with amoxicillin-clavulanic acid with a control group without antibiotic treatment. Molecular analysis of the digestive flora was performed over a 2-week experiment with FISH combined with flow cytometry (FC), using specific 16S rRNA targeted probes designed for the *Bacteroides-Porphyromonas-Prevotella*, *Clostridium coccoides-Eubacterium rectale*, *Clostridium histolyticum*, *Faecalibacterium prausnitzii,* *Enterobacteriaceae*, *Lactobacillus-Enterococcus*, groups and the *Bifidobacterium* genus.

Animal model. Eight-week-old female germfree C3H mice (INRA, Jouy en Josas, France) were maintained in sterile isolators and were given food sterilized by irradiation (Ro3 40 UAR) and water sterilized by autoclaving. Twelve mice had been gastrically intubated twice a week for 2 weeks with 0.5 ml of a suspension of feces from a gnotobiotic mouse harboring human fecal flora (INRA). For one group of six mice, each mouse was orally intubated with 0.4 ml of an amoxicillin-clavulanic acid solution (150 mg/kg of body weight) for 7 days, and one group of six mice was treated with saline and served as a control. The feces of each mouse were collected every day for molecular analysis of the flora and at days 2, 4, 9, 11, and 14 for enumeration of the total anaerobic flora.

Total anaerobic flora enumeration. The feces were removed, weighed, transferred within 10 min to an anaerobic chamber, and diluted. Total anaerobic flora was enumerated according to the method of Raibaud et al. (17). Bacterial counts were reported per gram (wet weight) of fecal content and expressed as $\log_{10}$.

FISH. Probes are detailed in Table 1. The EUB 338 probe was used as a positive control probe. The NON EUB 338 probe was used as a negative control probe (26). These control probes were linked at their 5′ ends with fluorescein isothiocyanate (FITC), and the group-specific probes were labeled at their 5′ ends with Cy5 (Cybergène). The technique was described by Rigottier-Gois et al. (18). The feces were removed, weighed, diluted in brain heart infusion broth (Difco) to give a 10-fold dilution, and homogenized. After decantation, aliquots of 0.2 ml were fixed with a fresh paraformaldehyde solution (4%) in phosphate-buffered saline (PBS) (130 mM NaCl, 3 mM Na_2HPO_4 $2H_2O$, 7 mM Na_2HPO_4 $12H_2O$ [pH 7.2]); one

* Corresponding author. Mailing address: Université Paris-Sud, Faculté de Pharmacie, Département de Microbiologie, 92296 Châtenay-Malabry Cedex, France. Phone: (33) 1 46 83 55 49. Fax: (33) 1 46 83 55 37. E-mail: anne.collignon@cep.u-psud.fr.

TABLE 1. 16S rRNA-targeted oligonucleotide probes

Probe (specificity)	Sequence from 5′ to 3′ end	OPD code[a]	Reference
EUB 338 (*Bacteria*)	GCT GCC TCC CGT AGG AGT	S-D-Bact-0338-a-A-18	1
NON EUB 338	ACA TCC TAC GGG AGG C	S-D-bact-0338-a-S-18	26
Bif 164 (*Bifidobacterium*)	CAT CCG GCA TTA CCA CCC	S-G-Bif-0164-b-A-18	13
Enter 1432 (*Enterobacteriaceae*)	GTT TTG CAA CCC ACT	S-G-Enter-1432-a-A-15	22
Chis 150 (*C. histolyticaum*)	TTA TGC GGT ATT AAT CT(C/T) CCT TT	S-*-Chis-0150-a-A-23	7
Bact 1080 (*Bacteroides*)	GCA CTT AAG CCG ACA CCT	S-*-Bacto-1080-a-A-18	3
Erec 482 (*C. coccoides-E. rectale*)	GCT TCT TAG TCA GGT ACC G	S-*-Erec-0482-a-A-19	7
Fprau 645 (*F. prausnitzii*)	CCT CTG CAC TAC TCA AGA AAA AC	S-*-Fprau-0645-a-A-23	24
Lab 158 (*Lactobacillus-Enterococcus*)	GGT ATT AGC A(C/T)C TGT TTC CA	S-*-Lacto-0158-a-A-20	8

[a] OPD code, Oligonucleotide Probe Database code.

volume with three volumes of fixative was incubated at 4°C overnight. The samples were then stored at −80°C until hybridization. The samples were pelleted by centrifugation (Eppendorf microcentrifuge; 12,000 rpm, 3 min, 4°C), washed, and resuspended with sterile PBS to remove residual fixative. Then, permeabilization was conducted with 1 ml of TE-HIS buffer (100 mM Tris-HCl [pH 8.0], 50 mM EDTA) and lysozyme solution (1 mg ml^{-1}) (Serva) in fresh TE-HIS buffer for 10 min at room temperature. Pellets were washed in 1 ml of PBS and then in 1 ml of hybridization buffer (900 mM NaCl, 20 mM Tris-HCl [pH 8.0], 0.1% sodium dodecyl sulfate, 30% formamide). The pellets were resuspended in 650 μl of hybridization buffer, and 50 μl from this suspension was distributed in each of 12 tubes, to which were added 4 μl of appropriate probe. Hybridization was conducted overnight at 35°C in darkness. Hybridization buffer (150 μl) was then added to each tube before pelletization by centrifugation as described above and resuspension in stringent washing (65 mM NaCl, 5 mM Tris-HCl [pH 8], 5 mM EDTA [pH 8], 0.01% sodium dodecyl sulfate) for 20 min at 37°C. After centrifugation at room temperature, the pellet was added to sterile PBS (200 μl). A volume of 100 μl from this suspension was added to 1 ml of FACS flow (Becton Dickinson) for data acquisition by FC.

FC. Data acquisition was performed using a FACSCalibur flow cytometer (Becton Dickinson) equipped with an air-cooled argon ion laser combined with a red-diode laser, as described in the work of Rigottier-Gois et al. (18). A total of 100,000 events were stored in list mode files. Results were expressed as cells hybridizing with the specific group-Cy5 probe as a proportion (percent) of the total bacteria hybridizing with the EUB 338-FITC bacterial domain probe.

Statistical analysis. The mean cell proportion was calculated using the results of duplicates. The Mann-Whitney (Wilcoxon) W-test and the Student test were performed using StatGraphics (Manugistics, Rockville, Md.) to determine whether there was a significant difference between the proportions of the bacterial groups at a confidence level of 95% ($P < 0.05$) in the two groups of mice.

The enumeration of the total microflora for the control mice was stable during the experiment: $2.4 \times 10^{10} \pm 1.4 \times 10^{10}$ CFU/g of fecal content. For the treated mice the level was also stable: $4.7 \times 10^{10} \pm 1.7 \times 10^{10}$ CFU/g (data not shown). With the EUB 338 FITC probe, the total for hybridized bacteria in the control mice was 67.3% ± 2.6% of 100,000 events, and for the treated mice it reached 69.1% ± 2.9% (Fig. 1A). The difference between the two groups was not significant with either technique. The antibiotic treatment did not quantitatively modify the level of the total flora assessed by culture or FISH.

The most abundant bacterial groups were detected with the *C. coccoides-E. rectale* and *Bacteroides-Porphyromonas-Prevotella* group probes, which represented the dominant flora. The level of the *C. coccoides* group was stable in the control mice (from 40.7% ± 1.6% to 45.6% ± 2.8%), but in the treated mice it decreased dramatically from the second day of treatment and stayed at a low level throughout the antibiotic treatment (3.9% ± 0.8%). When the treatment was stopped, the level increased (17.7% ± 4.7%) to reach 36% ± 1.8% at day 14 (Fig. 1B). The *Bacteroides* group in the control mice had a steady level of 35.9% ± 4.3%, whereas in the treated mice it increased from day 1 to reach 58.5% ± 0.4.5% at day 6. From day 9 the level decreased to 38.6% ± 5.7% to reach the same level as in the control mice at the end of the experiment (Fig. 1C). We can see in our animal model that amoxicillin-clavulanic acid disrupted the balance of the dominant anaerobic flora and that the *C. coccoides* group was very sensitive to amoxicillin-clavulanic acid. No *Enterobacteriaceae* were detected in the control mice, whereas they increased and were detectable in the treated mice from day 2 of administration. From day 8 they decreased to become undetectable from day 11 to the end of the experiment (Fig. 1D). Amoxicillin-clavulanic acid made the emergence of resistant *Enterobacteriaceae* possible. The *Faecalibacterium prausnitzii* (5), formerly *Fusobacterium prausnitzii*, and *C. histolyticum* groups totaled between 1.3% ± 2.1% and 0.4% ± 0.4%, respectively, in the control mice. With the treated mice, probes did not detect bacteria during the antibiotic treatment, signifying that these bacterial groups are sensitive to amoxicillin-clavulanic acid. One day after the antibiotic administration was stopped and until day 14, the level of these two groups was similar to that with the control mice (data not shown). The *Bifidobacterium and Lactobacillus-Enterococcus* probes did not detect any signals with our technique in either the treated mice or their control (data not shown).

Our animal model was used in this study to characterize the variations of fecal microflora with an antibiotic treatment assessed with FISH in combination with FC. Several studies have been carried out to investigate human fecal microflora in animal models with traditional techniques, and those authors report that the composition of the fecal microflora shows stability for a prolonged period of time (9, 10, 17, 21, 27). To our knowledge, no dynamic studies have yet been undertaken with

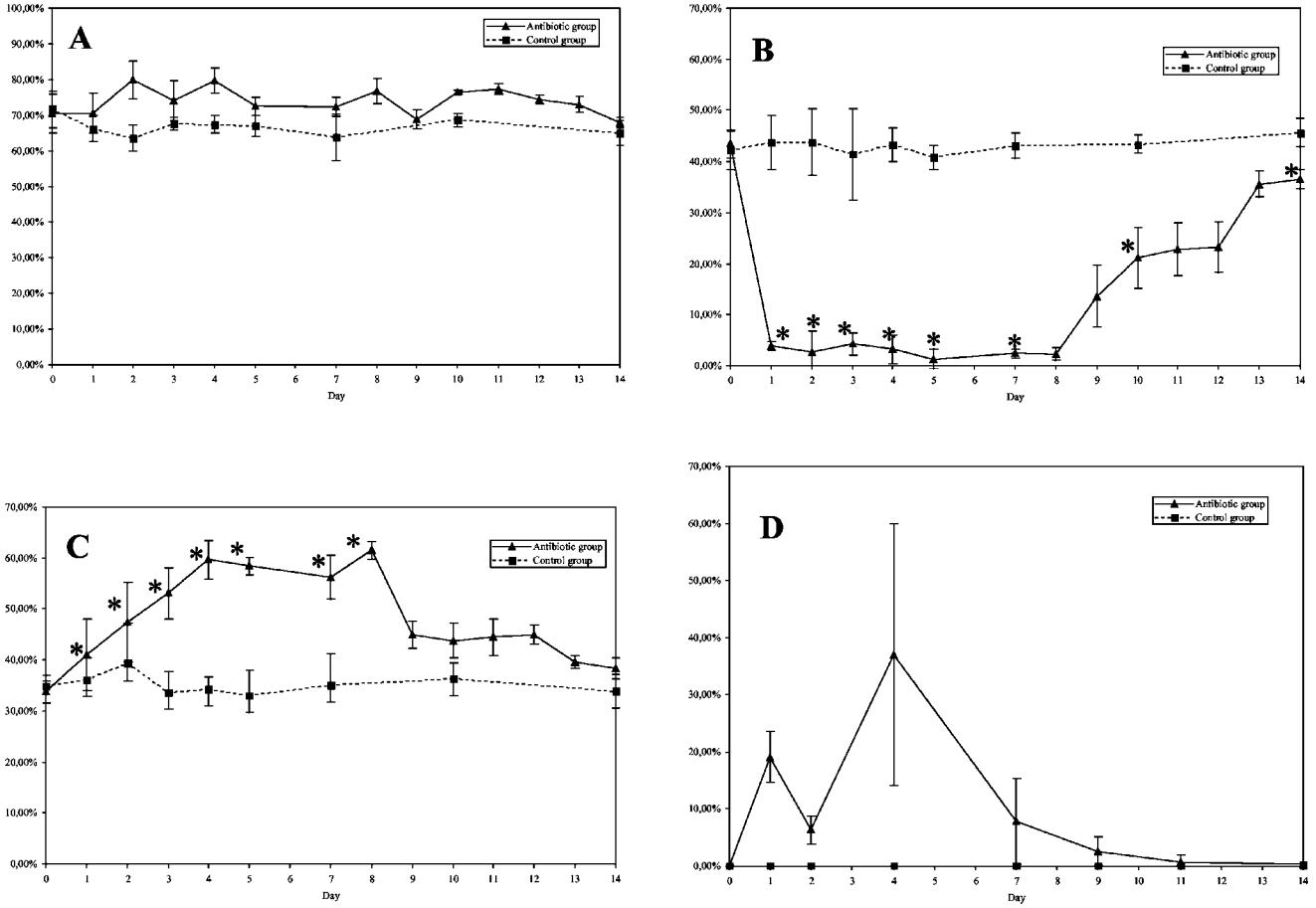

FIG. 1. Quantification of predominant groups of bacteria with FISH and flow cytometry detection using group-specific 16S rRNA oligonucleotide probes in two groups of human fecal flora-associated mice challenged with amoxicillin-clavulanic acid from day 1 to day 7 for the assay group (▲) and with saline from day 1 to day 7 for the control group (■). (A) Total anaerobic flora with the EUB 338 FITC probe. (B) *C. coccoides-E. rectale* group with the Erec 482 probe. (C) *Bacteroides-Porphyromonas-Prevotella* group with the Bacto 1080 probe. (D) *Enterobacteriaceae* group with the Enter 1432 probe. The data are means ± standard deviations of the means. An asterisk indicates that the *P* value is <0.05 between the two groups.

volunteers to evaluate the modification of human microflora under antibiotic treatment with amoxicillin-clavulanic acid with FISH associated with FC.

Several studies have been carried out with healthy volunteers with this molecular technique to evaluate only the composition of fecal microflora. In these studies the most abundant groups determined by FISH were the *C. coccoides* group, which represented 22 (18), 29 (7), 36 (19), and 22.8% (15) and the *Bacteroides* group, the level of which was 17 (19), 9 (18), and 20% (7), respectively. For the control mice, the composition of the microflora was consistent with results of these studies with healthy volunteers. No references in the literature mention the dramatic fall in the *C. coccoides* group or the simultaneous increase in the *Bacteroides-Porphyromonas-Prevotella* group with amoxicillin-clavulanic acid administration.

For human volunteers, the *F. prausnitzii* group was determined with FISH to be an important group, constituting 17% of the flora according to Rochet et al. (19), 11% for Rigottier-Gois et al. (18), and from 5 to 28% for Suau et al. (24). The *C. histolyticum group* was less than 1% according to Franks et al. (7). In our experiment the level of these two bacterial groups was lower than that for volunteers. In healthy volunteers the *Bifidobacterium* and *Lactobacillus* groups represented less than 1 and 0.6%, respectively, of total bacterial rRNA in fecal flora with FISH (22). For Harmsen et al. (8), Langendijk et al. (13), and Rigottier-Gois et al. (18), the level of *Bifidobacterium* was less than 3%. These two groups were at very low levels in humans, and they were not detected with our mouse model. In humans, facultatively anaerobic bacteria, such as *Enterobacteriaceae*, cannot be detected in normal fecal flora with FISH (4, 15, 18, 23), because they constitute a subdominant population and subsequently the level is not high enough to be detected with this technique. Studies with patients treated with amoxicillin-clavulanic acid demonstrate that *Enterobacteriaceae* of fecal microflora, evaluated by traditional methods, rise and become a dominant group of fecal microflora (12, 14, 16), constituting a risk of secondary septicemia. Similar results have been shown with mice (11), and they are consistent with our results assessed by FISH. Lode et al. (14) has shown with traditional techniques that *Escherichia coli* increases while the numbers of bifidobacteria, lactobacilli, and clostridia decrease. The fecal microflora of our animal model in the control mice

was very similar to that found in healthy volunteers, thus confirming the validity of our model. Use of the amoxicillin-clavulanic acid treatment makes it possible to demonstrate that the *C. coccoides-E. rectale* group decreases dramatically, whereas the *Bacteroides-Porphyromonas-Prevotella* group and the *Enterobacteriaceae* group increase, even though the total flora is not quantitatively modified.

In conclusion, the human fecal microbiota-associated mouse is an efficient model with which to study the ecology of intestinal flora. Indeed, healthy humans show variations in their microflora that are linked to diet and to external parameters. This model circumvents such variations and represents a possible standardized model for analyzing the flora during antibiotic treatment before human trials. The great advantage of molecular methods is to obtain information on specific groups of the dominant anaerobic flora, which are difficult to cultivate. In the future, new probes like those used for the *Atopobium* group (18) will be developed to detect new groups of uncultivable anaerobic bacteria. FISH combined with flow cytometry is a sensitive analytical technique and a high-throughput method that can rapidly detect changes in the composition of a complex microbiota.

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