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Selective Enumeration of *Bacteroides vulgatus* and *B. distasonis* Organisms in the Predominant Human Fecal Flora by Using Monoclonal Antibodies

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The genus *Bacteroides* represents about one-third of the isolates from human fecal samples. The proportions of the different species are difficult to estimate because there is no method for rapid identification of mixtures of anaerobes. Monoclonal antibodies against *Bacteroides vulgatus* and *B. distasonis* were prepared. They did not react with the other *Bacteroides* species of the *B. fragilis* group. These reagents allowed direct enumeration of *B. vulgatus* and *B. distasonis* organisms in human fecal samples. Anaerobic bacteria resistant to 1-h contact with air were enumerated in fecal human samples, a filter was layered on the colonies, and then *B. vulgatus* colonies were identified by an immunoassay performed with the prepared monoclonal antibodies. Healthy human adult volunteers were tested. Most of them harbored *B. vulgatus* at high levels, while the *B. distasonis* levels were always lower. Kinetic studies suggested that time variations for each volunteer were small. The simplified quantification of *Bacteroides* strains at the species level described here will prove useful in complementing our knowledge of the factors which may influence the predominant human fecal flora.

Studies performed during the past 20 years give an appreciation of the complexity of the normal colonic human microflora (for reviews, see references 5, 10, and 18). Although at least 400 distinct species of bacteria have been listed, five genera account for approximately 70% of the bacteria that can be cultivated (8, 11). The genus *Bacteroides* accounts for approximately 30% of the fecal isolates (18). Most of the *Bacteroides* strains of the predominant flora belong to the 10 species of the *Bacteroides fragilis* group (21). This genus undoubtedly plays an important role in the colonic ecosystem: digestion of diet polysaccharides and mucus (2, 12, 19, 20), involvement in antagonistic effects (22), modulation of bacterial toxin production (2), enhancement of local immune response (15), and probably many other properties not yet established. Since these bacteria can utilize dietary polysaccharides as a carbon source, their levels might be affected by the diet of the host (20). The importance of the *Bacteroides* spp. underlines the need for specific methods to quantify them in human fecal samples. *Bacteroides* sp. quantification will allow improvement of our knowledge of the different factors which may influence this predominant flora, such as diet modifications, bacterial recovery after antibiotic treatments, or spontaneous modifications. Genetic approaches for *Bacteroides* species detection based on the use of DNA probes (11, 12) or, more recently, 16S rRNA (9, 10) have been proposed. These methods of enumeration have not been utilized extensively, partly because of the limitations with genetics-based approaches of enumeration. Several broth culture media supplemented with additives such as bile, esculin, or antibiotics have been proposed for isolation of *Bacteroides* species from human stools (4). However, the counts of *Bacteroides* spp. in general are underestimated because colonic strains are partly sensitive to the proposed chemicals. Furthermore, each *Bacteroides* colony must be subcultured for further species identification.

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The aim of this study was to prepare monoclonal antibodies specific for some of the predominant *Bacteroides* species from human stools in order to develop a rapid and specific method for their enumeration in fecal samples. The antibodies obtained were used in a study undertaken to estimate the population levels of the corresponding *Bacteroides* species in feces of healthy volunteers.

Fecal samples were obtained from 50 adult healthy volunteers who gave consent to the study, which was approved by the local ethics committees. Exclusion criteria were digestive pathology and antibiotic ingestion during the period when samples were collected (except for one volunteer who ingested erythromycin [Ery 500], 1 g/day for 1 week). Undiluted fecal samples were kept in small closed containers and were processed within 3 h after collection. One gram of stool was homogenized in 10 volumes of saline. Afterward, the samples were diluted serially and plated on brain heart infusion–1% agarose medium (Difco Laboratories, Detroit, Mich.). The plates were kept on the workbench for 1 h before being placed in the anaerobic chamber. Colonies were counted after incubation at 37°C for 3 days in the anaerobic chamber.

Some *Bacteroides* strains came from a collection (A. Dublanchet, Villeneuve Saint Georges, France), but most of them were isolated by us from human fecal samples and identified by a rapid test (API32A; BioMérieux, Lyon, France). Only one strain per *Bacteroides* species was conserved from each stool. *Escherichia coli* and *Bifidobacterium* strains were isolated from human stools by us. Monoclonal antibodies were prepared as previously described (6), with the following modifications.

(i) Antigens used for immunization. A culture (2 days in brain heart infusion under anaerobic conditions) of *B. vulgatus* Pou6.3-G11 (isolated from the human digestive tract) was lysed by 15-min treatment with sodium dodecyl sulfate (SDS) (1% [wt/vol] final concentration) and incubated for 15 min at 50°C. Alternatively, a purified protein of *B. distasonis* was used. It was obtained as follows. A culture of strain FR-F3 (isolated from the human digestive tract) was lysed by a 15-min treatment with SDS (1% [wt/vol], final concentration) and boiled

for 5 min, then SDS-acrylamide gel electrophoresis (10% acrylamide) was performed, and the gel was stained with Coomassie blue. The part of the gel containing a protein band of 200 kDa was cut and homogenized in phosphate-buffered saline (PBS) (10 mM phosphate, 8% NaCl; pH 7). This dilution was used as an antigen.

(ii) Immunization procedure. Mice were immunized intraperitoneally with 20 μg of *B. vulgatus* or *B. distasonis* antigens mixed with Freund complete adjuvant. The subsequent challenges (once a month) were performed without adjuvant. Immunizations were continued until a high-level immune response was observed in sera, i.e., after 4 months. The last injection was made 2 days before spleen removal for the fusion process.

(iii) Screening test. Plates (Immunlo2; Dynatech Industries, Inc., McLean, Va.) were coated overnight at 4°C with the immunizing antigens at a 1/300 dilution in bicarbonate (0.1 M; pH 9.8), washed in PBS-Tween (PBS containing 0.5% [vol/vol] Tween 20), and incubated with hybridoma supernatants diluted 1/2 in PBS-Tween. After 2 h at 37°C and another wash, the plates were incubated for 2 h at 37°C with an anti-mouse immunoglobulin G coupled to peroxidase (diluted 1/1,000 in PBS-Tween) (Sigma, St. Louis, Mo.). After a second washing, the bound enzyme was revealed with tetramethyl 3,3′,5,5′-tetramethylbenzidine (TMB kit; Dynatech, Marnes-la-Coquette, France). The clones that gave a strong response were conserved for subcloning and amplification. Ascites fluids containing specific antibodies were tested by the filter technique described below. *Bacteroides* strains were enumerated on petri dishes (90-mm diameter). Plates with high numbers of isolated colonies were kept. Nitrocellulose filters (0.22-μm pore size, 82-mm diameter; Schleicher and Schuell, Dassel, Germany) were wetted (0.1 M bicarbonate, 3% SDS; pH 9.8) and layered on the colonies over a 30-min period at room temperature. The filters were washed with distilled water and incubated with PBS-Tween supplemented with 10% newborn calf serum (Gibco, Paris, France) for 0.5 h. They were then washed with PBS-Tween, covered with 10 ml of monoclonal antibodies diluted 1/500 in PBS-Tween, and kept overnight at 4°C. After a second washing, the filters were immersed in anti-mouse immunoglobulin G coupled to peroxidase (Sigma) diluted 1/1,000 in PBS-Tween and incubated for 2 h at 37°C. After another washing, the bound enzyme was revealed with TMB reagents. Positive colonies were blue.

Two different kinds of monoclonal antibodies, against *B. vulgatus* and *B. distasonis*, were raised. Initial screening of clones was performed by immunoassays with the immunizing antigens bound to plastic. Among the positive clones, those with the highest specificities against immunizing *Bacteroides* species were further analyzed by the selective enumeration technique described above. The specificities of our monoclonal antibodies were tested with the following numbers of strains: *B. fragilis*, 6; *B. vulgatus*, 11; *B. distasonis*, 5; *B. ovatus* and *B. uniformis*, 16 each; *B. caccae*, 5; *B. stercoris*, 4; *B. eggerthii*, 7; *E. coli*, 7; and *Bifidobacterium* spp., 10. Anti-*B. vulgatus* and anti-*B. distasonis* monoclonal antibodies did not react with heterologous *Bacteroides* spp. or with *E. coli* or *Bifidobacterium* species isolated from the digestive tract.

A method was developed and then used to enumerate *B. vulgatus* and *B. distasonis* organisms in human fecal samples. After serial dilutions and plating, the plates were kept in contact with air for 1 h or immediately introduced into an anaerobic chamber and incubated at 37°C; then total bacteria were counted, and *Bacteroides* organisms were selectively enumerated with the help of monoclonal antibodies (Table 1; Fig. 1). *Bacteroides* numbers were not modified by the 1-h air contact, but the numbers of total bacteria were reduced about 3- to 10-fold. Thus, for all human fecal sample analyses, *Bacteroides* organisms were enumerated after 1-h air contact in order to kill most of the very oxygen-sensitive anaerobes normally present in feces.

TABLE 1. Effect of exposure of plates containing diluted fecal samples to 1-h air contact prior to incubation in an anaerobic chamber

Volunteer no.	No. of bacteria ($\log_{10}$ CFU/g of stool)					
	Total		*B. vulgatus*		*B. distasonis*	
	Direct enumeration	After air contact	Direct enumeration	After air contact	Direct enumeration	After air contact
1	10.6	9.6	8.5	8.4	7.5	7.4
2	10.9	10.4	8.3	8.4	<6	<6
3	10.5	9.8	9.2	9.3	<6	<6
4	10.8	10.3	9.2	9.3	<6	<6

The average level of *B. vulgatus* in fecal samples of 50 healthy human volunteers was about 10^9 CFU/g of stools. Few volunteers (3 of 50) had no detectable *B. vulgatus*; one of them had a large number of *Bifidobacterium* organisms among the

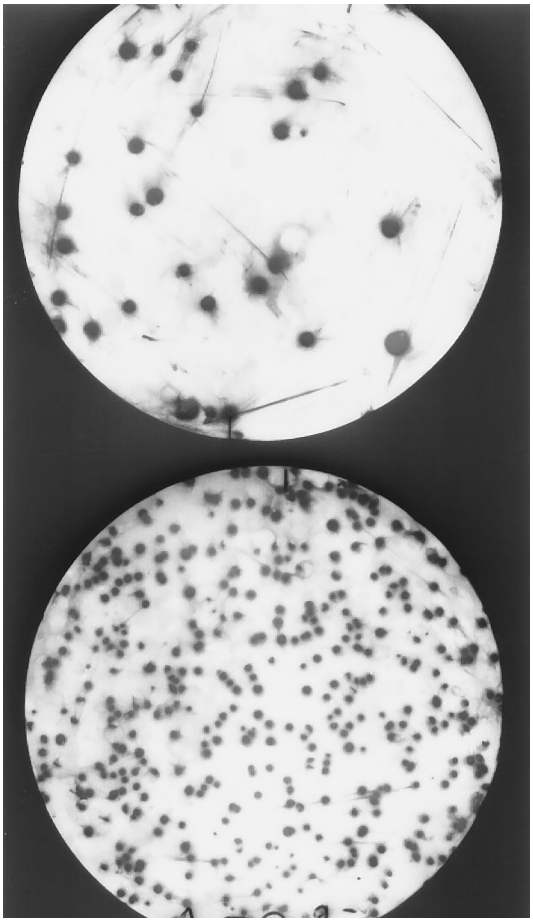

FIG. 1. Photograph of filters layed on petri dishes inoculated with 0.1 ml of 10^{-7} (top) and 10^{-6} (bottom) dilutions of one human fecal sample. Staining involving monoclonal antibodies against *B. vulgatus* was performed as described in the text. The number of *B. vulgatus* organisms in this sample was 3×10^9 CFU/g.

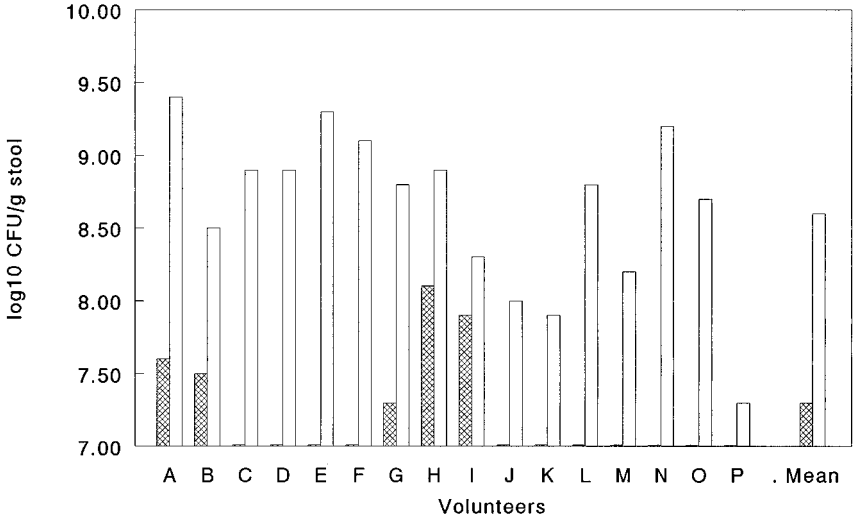

FIG. 2. Relative ratios of *B. vulgatus* (open bars) and *B. distasonis* (hatched bars) in human fecal samples. For each sample, the levels of the two bacteria were estimated. The average levels (mean) are also shown.

anaerobes resistant to air contact. More than 80% of the samples contained *B. vulgatus* at levels of $>10^8$ CFU/g. The detection limit was about 1 *B. vulgatus* (positive) colony per 1,000 colonies. The counts of *B. vulgatus* and *B. distasonis* in human fecal samples were estimated from fecal samples from 16 healthy volunteers (Fig. 2). The counts of *B. vulgatus* were always higher than those of *B. distasonis*. *B. distasonis* was rarely detected (11 of 16 subjects). A known quantity of *B. distasonis* was added to stool samples from four volunteers. The numbers of *B. distasonis* detected in the stools were close to the added quantities; differences were <0.5 $\log_{10}$ (i.e., less than a threefold decrease) (Table 2). Similar experiments of this type could not be performed with *B. vulgatus* since most of the volunteers harbored *B. vulgatus* at high levels.

Five or six serial samples were obtained from 10 volunteers over a 4-month period. Data for five representative volunteers are shown in Fig. 3. For each individual except one, an average level was apparent. One volunteer ingested erythromycin as a treatment for a sore throat before collection of sample 5. The *B. vulgatus* population in this subject became too low to be estimated for at least 6 months.

We prepared monoclonal antibodies against two *Bacteroides* species, *B. vulgatus* and *B. distasonis*, which are not closely related phylogenetically on the basis of their rRNA (7, 9). The antigens used were either total crude extracts of *B. vulgatus* or a protein described as species specific for *B. distasonis* (16). We obtained monoclonal antibodies that proved very specific for the *Bacteroides* species when used in the filter technique developed in this study. We did not use selective media, but the 1-h air contact killed the predominant anaerobic organisms which are sensitive to oxygen contact (5) and did not affect *Bacteroides* spp., which are resistant. This technique did not exclude facultative anaerobes or other anaerobes, such as *E. coli* and *Bifidobacterium* spp. However, *E. coli* is rare in the predominant microflora of healthy adults, and *Bifidobacterium* spp. are generally present at lower levels than *Bacteroides* spp. (8, 14).

TABLE 2. Addition of *B. distasonis* to fecal human samples

Volunteer	$\log_{10}$ CFU of *B. distasonis*/g of stools		
	In crude fecal sample	Added	Detected in stools
A	<6	8.1	8.2
B	<7	8.7	8.3
C	<7	7.6	7.3
D	8.3	9.4	9.7

Previous data (1, 3, 8, 13, 14) suggested that *B. vulgatus* was predominant in human fecal flora. Indeed, high levels of *B. vulgatus* were observed in most of the volunteer feces. For one volunteer, an undetectable level of *B. vulgatus* was related to an unusually high number of *Bifidobacterium* organisms. Benno et al. (1) found similar levels of *B. vulgatus* and *B. distasonis* in elderly Japanese persons. The level of *B. vulgatus* was not very different from that observed in our study. However, we have shown in this study that population levels of *B. distasonis* were generally far below those of *B. vulgatus*. Some fecal samples were supplemented with a known quantity of *B. distasonis*. Quantities similar to those added were detected in such stools, suggesting that no immediate killing of *B. distasonis* occurred and that this technique is relatively sensitive.

The variations of *B. vulgatus* levels among volunteers over a 4-month period were small, suggesting that the *B. vulgatus* levels were not highly influenced by minor variations of the ecosystem or the environment. Anecdotally, in the stools of one volunteer treated with an antibiotic for a short period, a decrease in *B. vulgatus* to an undetectable level was observed. The level did not increase during the following 6 months. In previous studies (8, 11), serial samples from a single donor were analyzed. It was found that substantial sample-to-sample variations existed. In our study, we did not observe such variations. Holdeman et al. (8) cultivated fecal samples on appropriate media and then picked 55 colonies in a randomized manner for each specimen and characterized clones. *Bacteroides* spp. represented about 10% of the clones. In our study, the identification approach was different: about 1,000 clones could be tested simultaneously. *B. vulgatus* or *B. distasonis* making up <1% of the total flora could be detected.

The enumeration approach described here uses fresh stools and has good sensitivity and precision, and the strains could be further characterized if necessary. However, genetic ap-

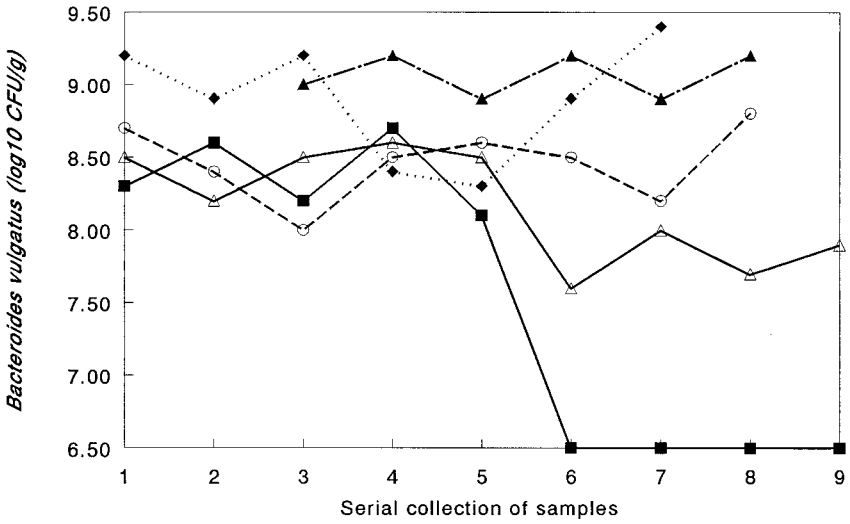

FIG. 3. Variations of *B. vulgatus* levels among volunteers in fecal samples collected in an 8-month period. Serial collection of samples was performed at intervals ranging from 1 week to 1 month. Each line corresponds to one volunteer.

proaches are also valuable because they do not measure exactly the same things. DNA probes allow precise *Bacteroides* quantitation, but the sensitivity is relatively low (11). PCR amplification increases the sensitivity, but the precision of quantitation is reduced (10). Both methods can be used on frozen samples, but they do not distinguish between living and dead bacteria and no further studies of the strains are possible.

Identification of species within a genus could be of great value for the study of gut microbial ecosystems. Some bacterial genera must be chosen as markers. We believe that *Bacteroides* could be one of them. The development of monoclonal antibodies coupled to fluorescein, for instance, may lead to new techniques, such as direct identification of *Bacteroides* cells in a sample with the help of image analysis systems. A similar approach with genetic probes containing colored markers is being developed.

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