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Comparison of viable cell counts and fluorescence in situ hybridization using specific rRNA-based probes for the quantification of human fecal bacteria

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Abstract

Conventional cultivation and fluorescence in situ hybridization (FISH) using 16S rRNA-based probes were compared for the enumeration of human colonic bacteria. Groups of common intestinal anaerobic bacteria were enumerated in slurries prepared from fecal samples of three healthy volunteers. To introduce variation between the samples, they were incubated for 48 h in batch culture (anaerobic) fermenters at 37°C, and pure cultures of *Bifidobacterium infantis*, *Clostridium perfringens*, or *Lactobacillus acidophilus* were added. Samples were taken from the fermenters at different times. Total anaerobes, bifidobacteria, bacteroides, clostridia, and lactobacilli were enumerated by both plating and FISH. The results showed that plate counts of total anaerobes, bifidobacteria, lactobacilli and bacteroides were approximately ten-fold lower than the corresponding FISH counts. Numbers of clostridia were higher using the plating method, probably because the clostridia probe used in FISH analyses was designed to only detect part of the genus *Clostridium*. The introduced variation in the methods could be detected by both methods and was comparable.

Keywords: 16S rRNA probe; Fluorescence in situ hybridization; Selective culture medium; Intestinal flora

1. Introduction

The gastrointestinal tract of humans contains large numbers of mainly obligatory anaerobic bacteria [1–3]. Highest cell densities of about 10^{11} g^{-1} of dry contents are present in the distal region of the colon. The cultivation and enumeration of different groups and species of these colonic bacteria are mainly carried out by culturing fecal samples on anaerobic selective media [4,5]. Usually, samples are diluted and plated on media thought to be specific for one group of bacteria. This classical principle has been used for years and the methods and the specific media are continuously refined. However, there are at least two fundamental problems which hamper the quantitative culturing technique. Firstly, this technique relies on the culturability of the samples [6–8]. Secondly, the accuracy of this method is dependent on selectivity of the medium. Often media are not truly specific and growth of unwanted bacteria affects the true counts, or media are so specific that only certain strains of a group grow on the plates [9]. This necessitates detailed phenotypic characterization which may be laborious and inaccurate.

Recent advances in rRNA-based molecular techniques make it possible to identify different bacterial populations in environmental samples without prior cultivation [8,10]. An example of this is the detection of bacteria in fecal samples [11–14]. In particular, fluorescence in situ hybridization (FISH) with specific 16S rRNA-based oligonucleotide probes has proven to be a useful tool for the identification of single cells within complex ecosystems [8,10,15–17]. Applying the FISH technique to human fecal samples to detect bifidobacteria demonstrated that this group of bacteria could be enumerated at least as accurately as by conventional cultivation techniques [16]. However, no comparative study has been carried out to analyze fecal samples by FISH using specific probes for the numerically

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important groups of anaerobic bacteria. Cultivation techniques can be used, despite their limitations, to measure the dynamics of subpopulations in the fecal flora. Whether FISH, with specific probes for these important groups, is also sensitive enough to detect subtle changes in the flora composition has not yet been addressed. We have compared conventional plating techniques on specific media, and FISH with specific oligonucleotide probes, to detect and enumerate different fecal subpopulations. Artificial variation was introduced in the samples by incubation for 2 days at 37°C and by the addition of pure cultures.

2. Materials and methods

2.1. Donors

The three healthy volunteers that donated fecal samples were aged 34 years (A, male), 20 years (B, female) and 24 years (C, female). They were on a normal western diet. None had taken antibiotics for at least 3 months prior to the study and none had a history of gastrointestinal disorders.

2.2. Sampling and cultivation of human feces

Fresh fecal samples (maximum lag time was 1 h) of the three volunteers were suspended in anaerobic phosphate buffer (pH 7.0) to give a final concentration of 10% (wet weight/volume). The slurry was homogenized and filtered to remove large particles and debris. The slurries were inoculated into 100 ml batch culture fermentation systems containing a medium as described [18]. Pure cultures of *Bifidobacterium infantis* or *Clostridium perfringens* or *Lactobacillus acidophilus* previously isolated from feces were added to three separate fermenters. When late logarithmic phase was reached 1 ml of culture was added. In one fermenter, a mixture of all three species was added and one fermenter served as control, so no additions were made. Samples were taken at the start of the experiment and after 48 h. In addition, one sample of the control was taken after 24 h. The samples were divided into two portions, of which one was fixed for FISH, labeled blind, stored on dry ice, and sent from IFR Reading to the Groningen laboratory for FISH analysis.

2.3. Enumeration of viable bacteria using plate culture

Ten-fold serial dilutions (from 10^2 to 10^{12}) were prepared from the fecal slurries, under an anaerobic atmosphere. The samples were plated in triplicate onto different specific media at all dilutions using the procedure and conditions of cultivation given by Wang and Gibson [4]. For total anaerobes, Wilkins Chalgren agar (Oxoid, Basingstoke, UK) was used. Lactobacilli were enumerated on Rogosa agar (Oxoid). For the enumeration of clostridia, Wilkins Chalgren agar was used supplemented with 8 mg l^{-1} novobiocin and 8 mg l^{-1} cholistin. Bifidobacteria were enumerated on MRS agar (Oxoid) supplemented with 20 mg l^{-1} nalidixic acid, 8.5 mg l^{-1} polymyxin B, 50 mg l^{-1} kanamycin, 35 mg l^{-1} iodoacetic acid and 25 mg l^{-1} 2,3,5-triphenyltetrazolium chloride. For *Bacteroides* a mineral salts agar (BMS) was used [19] supplemented with 10 mg l^{-1} nalidixic acid and 3 mg l^{-1} vancomycin. After 4 days incubation under anaerobic conditions at 37°C, colonies were counted on the selective media and at the appropriate dilutions. To confirm culture identity, different colonial morphotypes were examined using a combination of morphological, phenotypic and physiological criteria, such as cell morphology, Gram stain, API profiles, spores, the production of characteristic end products and presence of a specific enzyme (for the bifidobacteria) [20].

2.4. FISH analysis of the fecal samples

Samples of the slurries were diluted (1:3, v/v) in 4% paraformaldehyde in phosphate buffered saline (PBS) pH 7.2. The fixed samples were stored at −80°C until shipment on dry ice to Groningen. The samples were thawed only once and processed further. FISH was performed as described previously [16]. This procedure involved washing the sample with PBS, resuspending in PBS:ethanol (1:1) and storage at −20°C until the FISH experiment. Subsamples of the PBS:ethanol stock were hybridized at 50°C overnight in hybridization buffer containing 5 ng μl^{-1} fluorescein-labeled probe. Cells were washed at 50°C in hybridization buffer and filtered on a 0.2 μm polycarbonate filter (Millipore Corporation). Filters were mounted on a slide with Vectashield® (Vector Laboratories, Burlingame, CA, USA) and cells were counted visually using an Olympus BH2 epifluorescence microscope using a fluorescein- or a 4′,6-diamidino-2-phenylindole (DAPI)-specific filter. Ten to 25 microscopic fields were counted per assay. The coefficient of variation of the assay was determined by repeated processing and counting of a single fecal sample (0.5 g) and was found to be 0.20 for the DAPI stain and 0.18 for FISH analysis.

2.5. Oligonucleotide probes

Total cell numbers were counted using a nucleic acid staining with 4′,6-diamidino-2-phenylindole (DAPI). For the identification of bifidobacteria the Bif164 probe CATCCGGCATTACCACCC (probe sequence from 5′- to 3′-end) was used [16]. Lactobacilli and enterococci were enumerated with the Lab158 probe (GGTATTAGCA(T/C)CTGTTTCCA). This probe is specific for both lactobacilli and enterococci [21]. *Bacteroides* spp. were detected with a combination of two probes. The first probe, the Bfra602 (GAGCCGCAAACTTTCACAA) is specific for species of the *Bacteroides fragilis* subgroup. The second probe Bdis656 (CCGCCTGCCTCAAACATA) is spe-

cific for the species *Bacteroides distasonis*. For detection of *Clostridia* spp. the Chis150 probe (TTATGCGGTATTAATCT(C/T)CCTTT) was used. This probe is specific for the species of the *Clostridium histolyticum* subgroup. The specificity of the Bfra602, Bdis656 and Chis150 probes and their validation was described by Franks et al. [22].

3. Results and discussion

Two methods were compared to quantify the bacterial composition of fecal flora. Slurries were made from fecal stools of three healthy volunteers, and samples of these slurries were analyzed by plating onto selective agar media as well as by FISH. To introduce extra variation between the samples, the slurries were incubated at 37°C for 48 h in anaerobic batch fermenters. Pure cultures of *B. infantis* or *C. perfringens* or *L. acidophilus* were added to three separate fermenters. The results of all analyses are shown in Table 1. The viable counts of total anaerobes on the Wilkins Chalgren agar showed little variability. After 48 h incubation, there is a general trend for a decrease compared to the initial levels. The pure culture additions made little overall difference. Total cell counts as shown by DAPI staining generally were about three-fold higher than the viable counts. Although the numbers were constant, they showed a decrease after 48 h incubation in donors A and C. No decrease was observed in samples of donor B. However, this indicated that not all bacteria present in the samples were culturable on Wilkins Chalgren agar. Although this is a broad range growth medium, one particular genus may show improved growth compared to others. Bifidobacteria numbers, as determined on MRS medium, were relatively stable during the incu-

Table 1
The composition of the fecal flora samples of three human donors

		Total counts		Bifidobacteria		Bacteroides		Clostridia		Lactobacilli	
		Plating	DAPI	Plating	FISH	Plating	FISH	Plating	FISH	Plating	FISH
Donor A											
No additions	0 h	10.1	11.1	8.1	9.7	9.7	10.0	8.6	6.4	7.6	8.7
	24 h	10.6	11.0	8.5	9.8	10.0	10.0	7.1	6.5	7.2	8.2
	48 h	10.2	10.8	8.3	9.8	9.7	9.9	7.2	<5.8	6.9	8.8
+Bifidobacteria	0 h	10.7	11.1	9.4	10.0	9.5	10.3	8.4	6.8	7.4	8.2
	48 h	10.8	10.9	9.0	9.8	9.0	9.7	6.2	6.8	6.3	8.8
+Clostridia	0 h	10.3	11.1	8.0	9.8	9.0	10.3	8.9	7.4	7.3	8.1
	48 h	10.2	11.0	8.0	9.7	9.1	9.8	7.5	6.5	6.6	8.7
+Lactobacilli	0 h	10.3	11.2	8.4	10.0	9.2	10.1	8.2	6.5	8.2	8.0
	48 h	10.0	11.0	8.1	9.8	9.3	9.8	8.4	6.5	6.4	8.8
+Bif, Clos, Lact	0 h	10.9	11.0	8.6	9.7	9.5	9.9	9.2	7.3	7.7	8.1
	48 h	10.5	10.9	8.4	9.3	9.0	9.8	8.1	6.2	5.7	8.6
Donor B											
No additions	0 h	10.7	10.6	9.1	9.4	9.2	9.8	8.4	6.5	7.8	8.8
	24 h	10.7	10.9	9.2	9.9	9.5	10.3	8.5	6.5	7.4	8.3
	48 h	10.4	10.8	8.9	9.5	9.3	9.7	7.0	6.4	7.0	8.4
+Bifidobacteria	0 h	10.9	10.7	9.5	9.7	9.3	10.0	8.5	6.6	7.8	8.2
	48 h	10.3	10.7	9.2	9.5	8.9	9.7	6.1	<5.8	6.5	8.6
+Clostridia	0 h	10.1	10.9	8.8	9.9	9.4	10.3	9.1	7.0	7.9	8.5
	48 h	10.2	11.0	8.6	9.9	9.2	9.7	7.6	<5.8	6.7	8.7
+Lactobacilli	0 h	10.8	10.9	8.7	9.7	9.4	10.1	8.3	6.6	8.3	8.4
	48 h	10.6	10.8	8.4	9.6	9.4	10.0	8.4	<5.8	6.4	8.4
+Bif, Clos, Lact	0 h	10.9	10.8	9.2	9.7	9.2	10.0	8.8	7.1	8.1	8.6
	48 h	10.7	10.9	8.7	9.4	8.9	9.9	8.4	6.6	6.0	8.2
Donor C											
No additions	0 h	10.1	11.3	8.9	9.9	9.6	10.0	8.2	7.1	7.9	8.6
	24 h	10.7	10.9	9.5	10.0	9.3	10.2	8.2	6.8	7.4	8.1
	48 h	10.0	10.9	9.0	9.7	9.0	9.5	8.5	6.5	6.7	8.6
+Bifidobacteria	0 h	10.6	10.9	10.2	9.8	9.4	10.1	8.5	6.9	7.5	8.5
	48 h	10.4	10.9	9.5	9.6	8.9	9.6	6.3	6.1	6.5	8.5
+Clostridia	0 h	10.1	11.2	8.7	9.8	9.5	10.0	8.9	7.3	7.8	8.9
	48 h	10.8	10.7	8.6	9.6	9.2	9.7	7.5	<5.8	6.3	8.5
+Lactobacilli	0 h	10.2	11.2	8.9	10.0	9.7	10.2	8.6	6.7	7.6	8.3
	48 h	10.2	10.8	8.3	8.9	9.4	9.6	8.6	6.8	5.6	<6.0
+Bif, Clos, Lact	0 h	10.4	10.7	8.7	9.6	9.5	9.9	9.4	7.0	7.8	9.0
	48 h	10.5	10.8	8.6	9.7	9.1	9.9	8.3	6.1	6.0	8.6

FISH and plate counts are given as $\log_{10}$ g^{-1} of feces (wet weight). Plating was performed in triplicate. Using FISH, 10–25 microscopic fields were counted. The coefficient of variation for DAPI was 0.20 and for FISH 0.18.

bation period. As expected, numbers were markedly increased by the addition of *B. infantis*. The FISH counts of bifidobacteria using probe Bif164 were higher (up to 1 log) but also relatively stable. However, addition of *B. infantis* did not increase the FISH counts of bifidobacteria. This suggests that not all of the bifidobacteria present were culturable, since the addition of a pure culture increased the plate counts while the FISH counts remain unaffected. Numbers of *Bacteroides* enumerated on the BMS plates were five-fold lower than the FISH counts, using the FRA/DIS probe combination. Both methods showed little variation in response to incubation time or the addition of selected pure cultures. This may indicate that not all *Bacteroides* cells were culturable on the BMS plates. *Bacteroides* is a major component of the fecal flora which numerically was not affected by the additions of the selected species in this study. Clostridia counts decreased during incubation. This effect was most marked in the presence of added bifidobacteria. FISH counts using the Chis150 probe were considerably lower (1–2 log) than the plate counts. The FISH counts were often just above detection limit of 5.8 $\log_{10}$ g^{-1} (one cell per 20 fields) and sometimes even below the detection limit.

The Chis150 probe only detects part of the genus *Clostridium*, the *histolyticum* group. This comprises *C. perfringens*, *C. histolyticum*, *Clostridium putrificum*, *Clostridium butyricum*, *Clostridium carnis*, *Eubacterium multiforme* and other related species. The Chis150 probe does not detect the *Clostridium lituseburense* group, comprising *Clostridium difficile*, *Eubacterium tenue* and relatives. Therefore, our results indicate that most of the clostridia growing on the plates did not belong to the *C. histolyticum* subgroup. Numbers of lactobacilli enumerated on Rogosa agar were in the range usually detected by culture techniques in healthy volunteers [4]. The *L. acidophilus* addition initially increased counts in fermenters A and B only, but these counts decreased to the control levels at the end of the incubation. Enumeration of lactobacilli with the Lab158 probe gave generally more than ten-fold higher numbers of lactobacilli. The addition of *L. acidophilus* had no noticeable effect on the number of lactobacilli with FISH. The numbers of lactobacilli found using probing were higher than plate counts. The Lab158 probe detects not only lactobacilli, but also *Leuconostoc* and enterococci. However, this does not explain the high number of lactobacilli found, since most cells were rod-shaped. It may be possible that most lactobacilli present are nonviable cells which can be detected using FISH methods.

In conclusion, the results show that it is possible to detect variability in the numbers of major representatives of the human intestinal flora with both culturing and FISH although other media may give higher recoveries. However for accurate and fast identification the FISH methodology is probably more appropriate, since it is based on molecular markers. Added value of the approach is that stored samples can be used for analysis.

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