Variations of Bacterial Populations in Human Faeces Measured by FISH with Group-Specific 16S rRNA-Targeted Oligonucleotide Probes

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The human intestinal tract contains a complex ecosystem consisting mainly of obligatory anaerobic bacteria, usually referred to as the *normal flora*. The composition of this flora plays an important role in human health and disease. To measure the effect of treatments aimed at modulating the normal flora, we have developed fluorescent 16S rRNA targeted oligonucleotide probes for numerically important groups of bacteria in the human intestine. These probes have been used for whole cell fluorescent *in situ* hybridization (FISH) to study the composition of the human intestinal flora over time. To facilitate detection and enumeration, we have combined FISH with automated microscopic image analysis. Measurement of 48 samples can be done overnight. In this study we have focused on the number of bifidobacteria present in faecal samples of different age groups. The percentage bifidobacteria in faeces ranges from 0 to 78.9% depending on the age group. In each age group large variations were found. In faecal samples from formula-fed newborn infants we found individual differences ranging from 0 to 54.1% bifidobacteria and in faecal samples from elderly (77–91 yr) individual differences ranged from 0.2 to 41.3%.

Key words: FISH; anaerobic microflora; 16S rRNA probes; automated counting

INTRODUCTION

The human intestinal tract contains a complex ecosystem consisting mainly of obligatory anaerobic bacteria, usually referred to as the *normal flora* (3, 11). The composition of this flora plays an important role in human health and disease (5, 7, 13, 20). To understand the effects of treatments aimed at modulating the normal flora, accurate means for enumeration of the various bacterial populations are required. Conventional methods rely on cultivation of bacteria on selective media. However, many bacteria are difficult to culture or are unculturable, and often media are not truly specific (17, 19). Advances in the field of molecular phylogeny have made it possible to study bacterial populations by a culture-independent approach (2). Crucial for this was a ribosomal constituent: the 16S ribosomal RNA (16S rRNA). Each bacterial cell contains 10,000–60,000 ribosomes containing 16S rRNA and this means that detection of bacteria is facilitated using fluorescent *in situ* hybridization (FISH) with fluorescent oligonucleotide probes targeted at 16S rRNA (1, 2, 6, 9). Probes have been developed directed

at different phylogenetic levels (Domain, Family, Genus, Species) of the bacterial Kingdom and new probes are being developed with which it is possible to identify quantitatively a large number of different bacterial species of the gut ecosystem (1, 4, 8, 14, 15). These probes were used to study variations in bacterial populations in the human intestinal tract. A well-studied genus in the gastrointestinal tract is the genus *Bifidobacterium* (16). Bifidobacteria are numerically important in the gastrointestinal tract and their health-related properties are widely appreciated (18). In this study we will focus on the variations in the number of bifidobacteria in faecal samples of human volunteers. The changes over a period of 200–220 days were studied in adult volunteers and those in new-born infants were studied during the first 15–21 days of life. In addition, the percentage bifidobacteria in different age groups was determined. To facilitate detection and enumeration, we have combined FISH with automated microscopic image analysis (12).

MATERIALS AND METHODS

Faecal Samples from Newborn Infants

Faeces from newborn breast-fed and formula-fed infants was collected during the first 15–21 days of life. The faecal samples were fixed, using 0.5 ml sample in 0.5% cysteine-HCl storage buffer and 4.5 ml freshly

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prepared 4% paraformaldehyde in PBS. For this, 2 g paraformaldehyde is added to 30 ml Milli-Q followed by 100 μ l of 1 M NaOH. The solution is heated until handwarm (about 50°C, microwave) and the paraformaldehyde is dissolved slowly by gentle shaking. Next, 16.6 ml of 3-times concentrated PBS is added. The pH of the solution is adjusted to pH 7.2 and the volume is adjusted to 50 ml with Milli-Q. The solution is filtered through a 0.2 μ m filter and stored at 5°C. The solution should be used within 5 days. The fixed faecal samples were stored at -80°C.

Faecal Samples from Adult Volunteers over an 8-Month Period and Single Samples from Different Age Groups

A group of healthy volunteers ($n = 9$) ranging from 24 to 52 years provided fresh faecal samples twice a month for an 8-month period. In addition, faecal samples were collected from healthy volunteers (children and adults) who were not restricted in their normal western European diet and did not take antibiotics or other drugs known to influence the faecal flora composition for at least 4 weeks prior to sampling. The different age groups were: a group of children aged 2–3 years, a group of adults (20–52 years) and a group of elderly ranging in age from 77–91 years.

Portions (0.5 g) of each stool were suspended in 4.5 ml filtered (0.2 μ m pore size) PBS and vortexed with a few glass beads (4 mm diameter) for at least 3 min to homogenize the sample. The suspension was centrifuged at 700 $\times g$ for 1 min to remove debris. From the supernatant 1 ml was fixed in 3 ml freshly prepared 4% paraformaldehyde. After overnight fixation at 4°C the cells were stored in aliquots at -80°C until use. After thawing and careful mixing of the suspension, cells were diluted in 5% Tween-80. Suitable dilutions (e.g. 160 $\times$ for hybridization with the Bif164 probe) were applied to custom-made slides with square-shaped wells (1 $\times$ 1 cm). Slides were pretreated by soaking them in 0.1% gelatin, 0.01% KCr(SO₄)₂ $\cdot$ 12H₂O for 30 min at room temperature. Ten microliters was applied to each well and carefully spread over the total surface. After drying at room temperature, slides were fixed for 10 min in 96% ethanol. The slides with the fixed cells were kept in a dry atmosphere at room temperature until use.

OLIGONUCLEOTIDE PROBES

Fluorescein-labeled oligonucleotides were synthesized and purified by Eurogentec (Seraing, Belgium).

To enumerate all Bacteria, the bacterial probe S-D-Bact-0338-a-A-18 (Bact338): 5'-GCTGCCCTCCCGT-AGGAGT (1) was used. For enumeration of the bifido-

bacteria, the Bif164 probe was used: 5'-CATCCGGCA-TTACCACCC (14).

The design and validation of the Bif164 probe has been described elsewhere (14).

ENUMERATION OF BACTERIA BY FISH

Faecal Samples from Newborn Infants

Before analysis, the samples were transferred and concentrated into 1,200 μ l 50% ethanol in PBS. Depending on the amount of cells in the sample, part of the ethanol/PBS stock (usually 20 μ l) was hybridized with group-specific fluorescent 16S rRNA targeted oligonucleotide probes or stained with 4',6-diamidino-2-phenylindole (DAPI) for total cell counts as described before (4). Subsequently, samples were washed and filtered onto 0.2 μ m GTTP polycarbonate filters (Millipore, Etten-Leur, The Netherlands), mounted in Vectashield® (Vector Lab., Burlingame, CA) on microscope slides and enumerated using epifluorescent microscopy. Ten to 25 fields with a total of about 300 positive cells were counted for each sample.

Faecal Samples from Adult Volunteers

Cells from 1.5 ml of fixed suspension were washed twice in filtered PBS and resuspended in 300 μ l 50% (v/v) ethanol/PBS. The PBS:ethanol stocks were diluted 1:24 with 50°C hybridization buffer. The diluted cell suspensions (45 μ l aliquots) were added to 5 μ l 50 ng/ μ l oligonucleotide probes and hybridized overnight at 50°C. An aliquot of 5 to 50 μ l of this hybridized cell suspension was washed at 50°C for 30 min in 5 ml wash solution (hybridization buffer without SDS) before being filtered onto 0.2 μ m GTTP polycarbonate filters (Millipore, Etten-Leur, The Netherlands). For total cell

Fig. 1. Bifidobacteria in a faecal sample of a human volunteer detected with a FITC-labeled BIF164 probe. Scale bar, 5 μ m.

counts DAPI was added to the wash solution to a final concentration of 100 ng/ml. Filters were mounted on clean microscope slides with Vectashield®. Digital images of the slides, viewed with a Leica DMRXA epifluorescence microscope (Leica, Wetzlar, Germany), were taken with a Kodak Megaplug 1.4 CCD camera. An example of bifidobacteria in a faecal sample detected with the BIF164 probe is shown in Fig. 1. These images were analysed and fluorescent cells were counted using Quantimet HR600 image analysis software. Depending on the number of fluorescent cells, 10 to 30 microscopic fields were counted.

Faecal Samples from Different Age Groups

Faecal dilutions that had been fixed on the glass slides were hybridized with probes as follows. Probe dilutions (50 ng/μl) were made in Milli-Q and stored until use at -20°C. Prior to use they were further diluted in hybridization buffer of 50°C (20 mM Tris-HCl, 0.9 M NaCl, 0.1% SDS, pH 7.2) to a concentration of 10 ng/μl. Slides were incubated with these probe dilutions for 16 hr in a dark moist chamber at 50°C. They were rinsed with hybridization buffer of 50°C for 30 min. After briefly rinsing in Milli-Q, the slides were dried and mounted with 6 μl of Vectashield® on each well and a coverslip.

The slides were analyzed with an image analysis system consisting of a Quantimet 600HR-system, a Leica DM/RXA ultra-violet microscope equipped with a servo-controlled 8 slides-stage and a Kodak MegaPlus camera model 1.4. This system is connected to a second personal computer (Intelpentium 200 MHz, 64 MB RAM and 2.0 GB hard disk + Jazz-drive for datastorage)

which is mainly used for analysis of the images obtained by the Quantimet 600 system. Twenty-five fields were counted automatically in each well. Coefficients of variation of these counts below 0.10 were considered to be an indication of homogeneous distribution of cells in the well.

RESULTS AND DISCUSSION

Over the past few years, we have used several protocols to enumerate bacterial populations in faecal samples. The flora development in newborn infants was studied with visual counting of fluorescent bacteria. Cells were collected on filters and this precludes autofocusing under phase-contrast illumination. To monitor variations in different bacterial groups in 9 adult volunteers we still collected the cells on filters. However enumeration was done with the Quantimet image analysis software. Further studies showed that we lost not more than 2% of the total number of cells when the fixed cells were applied to glass. Custom-made slides with 6 wells (1 × 1 cm) were used for the next studies with samples from different age groups and this allows autofocusing under phase-contrast illumination and subsequent uv-illumination of the wells and automated counting of 25 fields in each well. Forty-eight faecal samples hybridized with one probe per sample can be processed overnight (12). Currently, miniaturization and more rapid autofocusing is investigated.

Figure 2 shows the development of bifidobacteria in faeces of 6 breast-fed babies measured with FISH. The relative numbers of the bacterial groups found in the samples are relative to the total number of cells counted

Fig. 2. Development of the bifidobacterial flora in the first 20 days after birth. Faecal samples of 6 breast-fed babies were analyzed with FISH. Probe Bif164 (14) was used to detect bifidobacteria. Total cell counts were obtained by DAPI-staining. The amount of bifidobacteria is given as percentage of the total number of cells.

Fig. 3. Development of the bifidobacterial flora in the first 20 days after birth. Faecal samples of 6 formula-fed babies were analyzed with FISH. The infants received the whey predominant infant formula Nutrilon Premium® (Nutricia, Zoetermeer, The Netherlands). Probe Bif164 (14) was used to detect bifidobacteria. Total cell counts were obtained by DAPI-staining. The amount of bifidobacteria is given as percentage of the total number of cells.

after DAPI staining of the cells. DAPI stains all cells containing nucleic acid in the sample. In this case, the Bact338 probe which detects all bacteria was not used. During the first days of life, bifidobacterial numbers were below 40%. But soon all bacterial flora's became dominated by bifidobacteria amounting to an average 78.9% of the total bacterial population and ranging from 62.9 to 91.0% of the flora on day 15 and 20. Using other probes, we could also detect streptococci, *E. coli* and *Bacteroides* in some of the babies (10). Figure 3 shows the development of bifidobacteria in faecal samples from 6 formula-fed babies. The overall picture of bifidobacteria development is much more diverse and there are large differences in the number of bifidobacteria in the samples of individual babies. The average percentage bifidobacteria was 32.2% with a range from 0 to 54.1%. Using other probes we can see that *Bacteroides* is present in higher numbers than in the samples from breast-fed babies (10). Very striking is the virtual absence of bifidobacteria (less than 0.01%) in one of the babies starting at day 10. Extremely low numbers of bifidobacteria were also seen in adults by Williams et al. (21). They studied the effect of adding a mixture of fructooligosaccharides to the diet on the human faecal flora. In two out of 10 volunteers the number of bifidobacteria was below the detection level (10^4 /g of faeces) they used for culturing. To gain insight into the normal biological fluctuations in bacterial populations in faeces, we have monitored variations in the bacterial composition of the faecal flora of 9 adult volunteers (age 24–52 yr) with a set of 4 probes (4). For

Bacteroides a combination of the Bfra602, Bdis656 probes was used. The Erec482 probe was used to detect the *Clostridium coccoides*–*Eubacterium rectale* group. The Bif164 probe and the Lowgc2P probe were used to quantify the bifidobacteria and the so far unculturable Low G + C #2 group (22), respectively. The Bact338 probe was used to detect all bacteria. The Bfra602/Bdis656 probe combination detected 20.4% of the total population, and Erec482 detected 28.9%. The Lowgc2P probe detected 11.8% whilst Bif164 detected only 3.4%. Together, they accounted for 2/3 of the total bacterial population. So far other probes only detect a minor part of the flora (less than 1%) (4, 8) and there is a clear need for new probes that can detect the remainder of the bacterial flora. Figures 4A and B show the variations in the bifidobacteria population over time. These volunteers were not on a restricted diet and thus the variations shown are those that commonly occur in a human population. The figure also shows that there are large individual differences. In some volunteers the bifidobacteria population stays at a relatively constant level (volunteers A, D and C) in others, large variations occur (e.g. E and G). Such differences should be taken into account when the effect of pro- and pre-biotics is studied. We also compared the number of bifidobacteria as percentage of the total population in faecal samples from different age groups (Table 1). The average percentage bifidobacteria is 78.9% and 32.2% in breast-fed and formula-fed infants (age 15–21 days), respectively. Then there is a steady decrease to 13.3% in children of 2 and 3 years of age. In a group of adults

Fig. 4. Percentage bifidobacteria in 9 adult volunteers (age 24–52 yr) over a period of 200–220 days. Probe Bif164 (13) was used to detect bifidobacteria. Total cell counts were obtained by DAPI-staining. A) volunteers, especially A, C and D in whose faeces the level of bifidobacteria was relatively stable, B) volunteers in whose faecal samples the bifidobacteria showed relatively large fluctuations.

Table 1. Percentage of bifidobacteria in different age groups determined with FISH (the number of bacteria hybridizing with the Bact 338 probe is taken as 100%).

	<i>n</i>	Mean	Range
Breast-fed infants (15–21 days)	6	78.9	62.9–91.0
Formula-fed infants (15–21 days)	6	32.2	0–54.1
Children (2–3 yr)	12	13.3	4.3–27.0
Adults (20–52 yr)	17	4.2	1.2–7.7
Elderly (77–91 yr)	14	10.1	0.2–41.3

we found 4.2% bifidobacteria and in elderly we found that bifidobacteria constitute 10.1% of the total bacterial population in faeces. However individual differences in the latter group are extremely large, ranging from 0.2 to 41.3%. It would be interesting to investigate the reasons for these differences and whether they are related to gastrointestinal motility, health status or diet.

In conclusion, the possibility to maintain a fixed diluted faecal sample at room temperature on a microscopic glass slide in a condition suitable for hybridiza-

tion studies at a later time opens new possibilities for quantitative studies with regard to bacterial flora composition. Applying FISH with a defined collection of probes not only directed at bacteria in faeces, but also at samples from within the intestinal tract will help to refine our knowledge about the spatial distribution and dynamics of gut bacteria and their interaction with the host in health and disease.

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