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Fluorescent *In Situ* Hybridization (FISH) as a Tool in Intestinal Bacteriology

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Fluorescent *in situ* hybridization (FISH) with 16S rRNA targeted oligonucleotides was used in combination with automated microscopy to determine the quantitative composition of the bacterial microflora in faeces of human volunteers. The percentage bifidobacteria and *Bacteroides* was determined in faecal samples of different age groups (12–20 d breast-fed, 1–3 yr, 18–52 yr, > 75 yr). The percentage bifidobacteria was 72.8, 10.0, 4.0 and 9.2%, respectively. The percentage *Bacteroides* was 1.3, 24.9, 20.6 and 5.9%, respectively. There are large individual differences especially in adults and even more in elderly. Comparison of the adult age groups in China and the Netherlands shows no significant differences in the percentage bifidobacteria and *Bacteroides*. However the percentage bacteria belonging to the *Clostridium coccoides–Eubacterium rectale* group was significantly higher in samples from China (31.4%) than in those from the Netherlands (19.9%). Individual differences were large. The effect of oral treatment with metronidazole on the bacterial composition in faecal samples of one volunteer was investigated with several probes. There is a 3-fold decrease in the total number of bacteria and the results show that there is a large increase of lactobacilli–enterococci and an increase of bifidobacteria. *Bacteroides* remains unaffected. The results show that automated FISH is an excellent tool to study intestinal bacteriology in response to modulation.

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

INTRODUCTION

The microbiota of the intestinal tract is an essential part of an ecosystem that plays an important role in health and disease *(5, 6, 11, 21)*. The highest number of bacteria, circa 10^{11} per gram of dry contents, is present in the colon *(12, 18, 19)*. Most (> 99%) of these bacteria require an anaerobic environment for growth and multiplication. What is the bacterial composition of this intestinal ecosystem? How many different bacteria are there, how do they interact and what are their activities? Those are questions that need to be addressed to understand this ecosystem. In this study we will focus at the quantitative bacterial composition of faecal samples of healthy volunteers.

The classical method to determine the quantitative composition of bacteria in such samples is culturing on suitable growth media. The sample is diluted and plated on a specific medium. The bacterial count of the original sample is then determined by multiplying the number of colonies that develop by the degree of dilution. There are two important problems using this technique. Firstly, the bacterial count depends on the culturability of a bacterial species. For many years it has been explicitly stated by several microbial ecologists (summarized in ref. 22) that as long as not all bacteria can be cultured it will be impossible to define the microbial composition of the sample. Not all bacteria can be cultured and therefore this will lead to an underestimation of the quantitative contribution of certain genera. Secondly, specific media are not truly specific and certain bacterial species may be counted more than once on different 'specific' media. This may lead to an overestimation of the quantitative contribution of certain genera. The net result is an inaccurate picture of the composition of the gut flora and this method is therefore not suitable to study population dynamics in the intestinal tract.

Advances in the field of molecular phylogeny have made it possible to study bacterial populations by a culture-independent approach. Crucial for this was a ribosomal constituent: the 16S ribosomal RNA (16S rRNA). Ribosomes play a key-role in the protein synthesizing machinery of a cell and each cell contains 10,000–60,000 ribosomes. A bacterial ribosome consists of a 50S and a 30S subunit. These subunits consist

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of proteins and RNA. The 30S subunit contains 21 different structural proteins and a 16S rRNA molecule. An average bacterial 16S rRNA molecule contains 1,500 nucleotides. The 16S rRNA molecule has become an important tool in molecular phylogeny studies *(26)*. Comparison of sequences of different bacterial 16S rRNAs shows that the molecule contains segments with different degrees of variability. This made it possible to construct phylogenetic trees and it revealed evolutionary relationships between species *(2, 20)*. The different degree of variability also lead to another application of the sequence information. Currently, more than 17,000 16S rRNA sequences are available and this allows the design of DNA-probes, usually consisting of 18–27 nucleotides, that hybridize with a particular sequence in the 16S rRNA molecule.

We have probes available directed at different phylogenetic levels (Domain, Family, Genus, Species) of the bacterial Kingdom. With these, it will be possible to identify quantitatively and qualitatively a large number of different bacterial species of the gut ecosystem. To this end, we will apply fluorescent *in situ* hybridization (FISH) with fluorescently labeled 16S rRNA targeted oligonucleotides *(7, 10, 15)*. Quantification of positively hybridized bacteria is usually performed by means of visual counting. Because this is a time-consuming process and heavily depends on the skills and experience of the technician, only moderate levels of reproducibility are reached *(15)*. To overcome these problems, an automated microscopic counting procedure was developed *(13)* comprising (i) a software-program for fully automated microscopic counting of whole-cell hybridized gut microflora and (ii) a validation of the automated counting procedure. The source code of the software was written using the QUIPPS-interpreter supplied with the Leica Q550 image analysis system. The reason for this choice is that applications built in the QUIPPS-environment can be used to control the Leica DM/RXA ultra-violet microscope while, simultaneously, images acquired with the charge-coupled device (CCD) can be obtained and processed. The automated system was further improved and the accuracy of quantification of automated FISH and culturing was compared. With the improved automated FISH method, the number of *Bacteroides* and bifidobacteria in human faecal samples was studied in relation to age. Faecal samples of four age groups (12–20 d breast-fed, 1–3 yr, 18–55 yr, > 75 yr) were investigated. In addition to differences in bacterial composition related to age, differences in faecal microflora in different countries may exist. We compared the percentage of three groups of intestinal bacteria i.e. *Bacteroides*, bifidobacteria and the *C. coccoides–E. rectale* group in healthy volunteers (age 18–55 y) in China and in the Netherlands. An important aspect of microflora modulation is the effect of antibiotic treatment and subsequent recovery of the bacteria microflora. This was investigated in one volunteer who received oral treatment with metronidazole. The effect on six bacterial groups was quantitatively measured before, during and after treatment.

MATERIALS AND METHODS

Faecal samples. Faecal samples were collected and fixed as described earlier *(23)*. From one volunteer (age 29 yr) 4 faecal samples were collected during 14 days preceding a one-week oral treatment with 500 mg metronidazole (3 times/day). Three samples were collected during treatment and 5 samples till 6 weeks after treatment.

OLIGONUCLEOTIDE PROBES

Fluorescein-labeled oligonucleotides were synthesized and purified by Eurogentec (Seraing, Belgium). Only validated probes were used in this study. To enumerate all Bacteria, the bacterial probe (Bact338): 5'-GCTGCCTCCCGTAGGAGT *(1)* was used. For enumeration of bifidobacteria, the Bif164 probe was used: 5'-CATCCGGCATTACCACCC *(15)*. Other probes used were: a digenus-probe specific for *Bacteroides* spp. and *Prevotella* spp. (Bac303) 5'-CCAATGTGGGGAC CTT *(17)*; a probe specific for the *C. coccoides–E. rectale* group (Erec482): 5'-GCTTCTTAGTCAR GTACCG *(7)*; a probe specific for the *Atopobium* cluster (Ato291): 5'-GGTCGGTCTCTCAACCC *(10)*; a probe designated as ELGC for the low G + C #2 group *(25)* to which one extra nucleotide (G) was added resulting in the sequence: 5'-GGGACGTTGTTTCTG AGT; and a probe that detects the lactobacilli–enterococci group (Lab158): 5'-GGTATTAGCA(C/T) CTGTTTCCA *(9)*. To account for non-hybridizing bacteria a 4',6-diamidino-2-phenylindole-stain (DAPI), which stains all DNA containing objects, was also included.

RESULTS AND DISCUSSION

Automated Counting Procedure

Since the publication of the automated microscope-based method for enumeration *(13)*, improvements have been made. They comprise (i) upgrading of the hardware configuration and optimization of the software and (ii) miniaturization and further reduction of analysis

time. With respect to (i) the improved method makes use of the Leica Q550 image analysis system, a Kodak MegaPlus camera model 1.4 and a servo-controlled Leica DM/RXA ultra-violet microscope. The performance of the method was validated by measuring 20 faecal samples from healthy human volunteers using the Bif164, Bact338 (universal probe), Erec482, Bac 303 probes and the DAPI-stain. A series of performance-parameters was quantified and the results are listed in Table 1. The combined effect of upgrading the hardware and software-optimization results in a near doubling of the performance of automated counting.

With respect to (ii) new microscopic slides were designed to further improve the performance. The original system comprised slides with 6 square wells with a surface area of 1 cm^2 on an 8-slides stage. The new slides contain 18 wells in a 3 × 6 configuration. Each separate well has a surface area of 25 mm^2. The average time to count one well is 16.5 min. Reduction of the counting time may be achieved by: a) refraining from continuous refreshment of the screen (reduction of 4 min/well); b) finishing the routine of counting 25 fields when the coefficient of variation (CV) is less than 10% with at least 10 fields and 300 bacterial cells counted. The reduction in time will vary; c) reduction of waiting time between movement steps of the motorized stage will result in a reduction of 3 min/well. Counting of the 18-wells slides will then take approximately 23–24 hr. A four-slides stage may then be preferred over an 8-slides stage to diminish possible bleaching of fluorescence.

Table 1. Performance of the automated counting procedure.

Parameter	Value*	New value
Mean focusing time	40 sec	20 sec
Mean image analysis and storage time	10 sec	5 sec
Mean time needed for change of wells	5 sec	1 sec
Mean time needed for change of slides	5 sec	1 sec
Fraction of fatal out-of-focus incidents	0.01	< 0.01
Number of images per well	25	25

*Values mentioned in ref. *(13)*.

Table 2. Comparative assessment of FISH and culturing.

Target	Method	CV_{assay}*	$CV_{Individual}$**	F-C***
Bacteria	FISH	0.12	0.53	0.000
	Culture	0.47	0.01	
Bacteroides	FISH	0.07	0.66	0.001
	Culture	0.30	0.65	
Bifidobacteria	FISH	0.14	0.64	0.881
	Culture	0.22	0.56	

*Coefficient of variation (CV) due to the process of slide preparation.
**Coefficient of variation (CV) due to biological differences between volunteers.
***Comparisons performed using Wilcoxons rank sum test. F-C = FISH compared with culture.

Comparison of Selective Culturing and Automated FISH

Enumeration of faecal microflora with FISH with the improvements mentioned in Table 1 was compared with selective anaerobic culturing. The performance of FISH was validated using a set of 4 fluorescent oligonucleotide probes: a universal probe for the detection of all bacterial species (Bact338), one probe specific for *Bifidobacterium* spp. (Bif164) and a digenus-probe specific for *Bacteroides* spp. and *Prevotella* spp. (Bac303). For the culturing-assays the following selective media, which are presumed to have about the same selectivity as the probes used in FISH, have been used: Wilkens-Chalgren agar for the total number of anaerobes, BMS-agar for the number of *Bacteroides* and BF-agar for the number of bifidobacteria. To quantify the assay-error, one faecal sample was measured twenty times using each separate probe or growth medium. The error in each of the datasets (i.e. the assay-error) was expressed as the coefficient of variation (i.e. standard deviation / mean). Subsequently, faecal samples of twenty volunteers were measured following the same procedure in order to quantify the error due to individual-related differences in gutflora composition. The composite error measured this way was then corrected by arithmetical subtraction of the assay-error. The remaining value, or the interindividual error is also expressed as a coefficient of variation. The results of the twenty different volunteers (per target group) were compared with each other. In Table 2, the results are listed. From these results it can be concluded that the assay-errors of the FISH method are smaller than the assay-error for culturing. The relatively low value of the assay-error of the FISH method indicates a large discriminating power to distinguish real differences between volunteers. This seems especially true when total numbers of bacteria are determined. When comparing the actual numbers obtained with the two methods, it appears that only the numbers of bifidobacteria are significantly the same when using culture or FISH (Wilcoxons rank sum test: 0.881). The total number of bacteria is not significantly identical. This is probably due to the fact that only a fraction of the total number of bacteria present in the flora is vital. The number for

Bacteroides obtained with culture and FISH is also not identical. This may very well be due to the differences in specificity between the probe (which also detects *Prevotella*) and the culture medium.

Age-Related Differences in Bacterial Composition of Faecal Microflora

In an earlier study *(23)*, differences in the percentage bifidobacteria present in faecal samples of different age-groups were investigated. In the present study the number of volunteers in the age-groups 1–3 yr and 18–55 yr was larger and the percentage *Bacteroides* was also determined. The results are summarized in Table 3. The results show that bifidobacteria are the predominant bacteria (72.8% of the total number) in faecal samples of breast-fed newborn infants. From age 1–3 yr this percentage decreases to 10.0% and still further decreases between 18 and 52 yr to 4.0%. For *Bacteroides*, the picture is quite different. They do not play a numerically important role in the new-borns (2.8%), but they increase rapidly to 24.9% between 1–3 yr and stay at approximately the same level between 18 and 52 yr (20.6%). However, at age 75 yr or more the percentage of *Bacteroides* shows a large decrease to 5.9%. As shown by the standard deviations in Table 3, there are large individual differences especially in adults but even more in elderly. For bifidobacteria in faecal samples of elderly, percentages ranged between 0.1 and 54.9%. For *Bacteroides* the range in samples from elderly was 0.4 to 32.8%. With the existing probes, approximately 2/3 of the total microflora could be detected in adults (18–55 yr). The larger part of the faecal microflora of elderly remains undetected with these probes.

Table 3. Percentage of bifidobacteria and *Bacteroides* in different age groups determined by FISH (DAPI count = 100%).

n	Age	Bifidobacteria	*Bacteroides*
6	12–20 d (breast-fed)	72.8 (16.0)	1.3 (2.8)
23	1–3 yr	10.0 (5.5)	24.9 (12.7)
41	18–55 yr	4.0 (4.4)	20.6 (10.3)
15	>75 yr	9.2 (14.7)	5.9 (8.2)

s.d. in parentheses.

Differences in Bacterial Composition in Faecal Microflora in China and the Netherlands

So far our quantitative studies were performed with faecal samples from the Netherlands. To create a solid base for future comparative microflora studies involving faecal samples from various countries, we proposed to perform a global study (XIIIth International Symposium on Gnotobiology, June 1999, Stockholm). Collaborating groups were provided with gelatin-coated glass slides and an extensive protocol for fixation and application of faecal dilutions from 18 volunteers (18–55 yr) to the slides. Slides were sent to our laboratory for subsequent hybridization, image analysis and processing. This study is not finished yet, however here we will present preliminary results from the first

Table 4. Effect of 1 week treatment with metronidazole (500 mg p.o. 3 times daily) in one healthy volunteer on the bacterial composition.

Days	Bifidobacteria (%)	Lactobacilli–enterococci (%)	Atopobia (%)	Low G+C#2 (%)	*C. coccoides–E. rectale* (%)	*Bacteroides* (%)	Rest (%)
1	3.1	0.1	5.0	2.2	11.8	3.8	73.8
3	2.1	0.1	4.3	1.0	13.6	7.7	71.0
7	3.9	0.1	3.2	1.4	23.1	12.5	55.6
10	8.9	0.1	4.3	2.3	15.0	5.4	63.7
17 (d2 treatment)	8.1	0.3	5.5	2.0	21.2	8.2	54.2
19 (d4 treatment)	30.2	8.1	6.6	3.9	23.9	12.2	14.6
22 (d7 treatment)	25.8	9.7	5.3	9.7	21.5	17.9	9.9
3 (after treatment)	8.1	2.2	4.3	2.5	15.6	11.7	55.4
7	8.9	0.4	4.7	1.9	8.2	3.7	72.2
14	3.7	0.1	4.8	2.8	15.2	7.1	66.3
28	1.9	0.1	3.6	1.4	14.9	8.0	70.0
42	4.9	0.2	3.6	1.0	21.4	8.9	59.8

The following probes were used: Bif 164 for bifidobacteria; Lab 158 for lactobacilli–enterococci; Ato 291 for the *Atopobium*-cluster; ELGC for the low G + C #2 group; Erec 482 for the *C. coccoides–E. rectale* group; Bac 303 for *Bacteroides* spp. and *Prevotella* spp. Total cell counts were obtained by DAPI-staining. The amount of bacteria is given as percentage of the total number of cells. Rest is the group of bacteria that does not hybridize with the aforementioned probes.

samples that were collected in a pilot-study. The percentages bifidobacteria, *Bacteroides* and bacteria belonging to the *C. coccoides–E. rectale* group were determined in 23 samples from China. The results are shown in Table 4. The percentage bifidobacteria in samples from China is 3.1% and this is slightly lower but not significantly lower than in samples from the Netherlands. The percentage *Bacteroides* is 24.1% and this is slightly higher, but not significantly higher, than in samples from the Netherlands. The percentage bacteria belonging to the *C. coccoides–E. rectale* (*Erec*) group is 31.4% in samples from China and this is significantly lower than the 19.9% in samples from the Netherlands. Whether a specific part of this heterogeneous group of bacteria is more predominantly present in samples from China is not yet known. Individual differences in the *Erec*-group are very large. They range from 8.3 to 70.7% in the samples from China and from 6.5 to 36.0% in those from the Netherlands. Diet questionnaires of the volunteers will be analyzed to investigate a possible relation between individual microfloras and diet.

Effect of Metronidazole on Bacterial Composition of Faecal Microflora of One Volunteer

Metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] was first used in 1959 for the treatment of *Trichomonas vaginalis* infections. However it was shown to be useful for a variety of other infections *(8)*. In order to kill susceptible cells, reduction of the nitro group is required. The spectrum of activity is limited to those organisms capable of anaerobic metabolism. Conflicting results were reported on the effect of metronidazole on the anaerobic bacterial microflora of humans. Dion et al. *(4)* reported a reduction in the number of *Bacteroides*, while other authors reported no change *(3, 14, 17)*. Metronidazole was also reported to have little effect on the total anaerobic faecal microflora and the number of bifidobacteria increased *(17)*. Consensus appears to exist with regard to the increase in the number of enterococci *(3, 4, 14, 16, 24)*. All these studies were performed by culturing. Since automated FISH is more accurate to determine differences in bacterial numbers (see Table 2), we decided to reinvestigate the effect of oral treatment with metronidazole in one healthy volunteer. The total number of bacteria as determined by staining with DAPI decreased from 8.0×10^{10} per gram of wet faeces immediately before treatment to 2.6×10^{10} per gram at day 7 of treatment and came back to 7.3×10^{10} per gram after treatment. Such changes would have been difficult to determine accurately by culturing. The results for specific groups of bacteria are shown in Table 4 as percentage of the total number of cells. These percentages reflect composition and not numbers. The largest changes are found in the percentage bifidobacteria and lactobacilli/enterococci. A six-fold increase in the percentage bifidobacteria and a 9-fold increase in the percentage lactobacilli/enterococci. The latter group was shown to consist mainly out of enterococci by culturing of faecal dilutions on esculin agar. The composition does not give information on the actual numbers. The cell numbers at day 7 of treatment divided by those before treatment (average of four samples) were: bifidobacteria, 1.8; lactobacilli/enterococci, 31.9; atopobia, 0.4; ELGC-group, 0.3, *C. coccoides–E. rectale* group, 2.1; *Bacteroides*, 0.8. With regard to the bifidobacteria, lactobacilli–enterococci and *Bacteroides* these results are in agreement with those obtained by culturing *(3, 14, 16, 24)*. The atopobia and the ELGC-group are susceptible to treatment and the *C. coccoides–E. rectale* group shows a 2-fold increase in numbers although this does not affect the composition (see Table 4). The rest group of bacteria (those bacteria that are not detected by our probes) present in the faeces of our volunteer is affected by metronidazole treatment since it decreases from an average of 60% before treatment to 9.9% at day 7 of treatment and returns back to 60% after treatment.

CONCLUSIONS

During life, the percentage of bifidobacteria in human faecal samples decreases from 72.8% in breast-fed newborn infants to 4.0% in adults (18–52 yr). In elderly this percentage increases to 9.2%. The percentage *Bacteroides* shows a steady increases from 1.3% in breast-fed new-born infants to 20.6% in adults (18–52 yr) and decreases to 5.9% in elderly. Comparison of the same bacterial groups including the *C. coccoides–E. rectale* group in faecal samples from adults in China and the Netherlands showed no differences in percentages bifidobacteria and *Bacteroides*. The percentage of the *C. coccoides–E. rectale* group was significantly higher in the samples from China. There are large individual differences in the bacterial composition of all these faecal samples.

The effect of metronidazole treatment on the composition of the faecal microflora and the reestablishment of the microflora present before treatment could be measured accurately with a number of 16S rRNA-targeted probes. These results show that automated FISH is an excellent tool to study intestinal bacteriology in response to modulation.

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