Characterization of human faecal flora by means of an improved fluoro-morphometrical method

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SUMMARY

The use of polyclonal antibodies in differentiating between samples of faecal microflora derived from ten healthy volunteers was assessed. The distribution of FITC-labelled antibodies (of the IgA, IgG and IgM isotype) over 144 morphologically distinct subsets of faecal bacteria ('morphotypes') was quantified by means of digital morphometry and quantitative immunofluorescence. Furthermore, a new dataprocessing algorithm was developed which makes objective quantitation of the antibody distributions over the faecal morphotypes possible. The results of this study imply that the antibody binding capacity of faecal morphotypes is a unique characteristic of faecal microflora.

INTRODUCTION

The composition of the faecal microflora is supposed to be an important parameter in the aetiology of nosocomial infections [1], rheumatoid arthritis [2], Crohn's disease [3] and colon carcinogenesis [4]. Colonic overgrowth by resistant bacteria during antimicrobial chemotherapy [5] reveals that the colonization resistance [6, 7] of the gut is mainly determined by the anaerobic faecal microflora.

In order to assess the composition of the faecal microflora, the following strategies are employed most often: anaerobic culture [8], measurement of the activity of enzymes produced by faecal bacteria [9] and morphological analysis of faecal microflora by means of light microscopy [10].

Since human faeces may contain as many as 400 different anaerobic bacterial species, culturing of faecal bacteria is very laborious and time-consuming. Quantification of the activity of bacterial enzymes can proceed rapidly and a reasonable correlation between enzyme activity and the anaerobic culturing results has been reported [11]. However, because different bacterial species may produce the same enzymes this method is less well suited for characterization purposes. The microscopic morphology of faecal microflora can be analysed visually [10] and automatically [12]. Visual morphological analysis is laborious and may be inaccurate as a result of its inherent subjectivity. On the contrary, automatic morphological analysis is fairly fast and accurate, and has therefore been applied clinically to study the effect of antibiotic treatment on faecal flora

[13], although the sensitivity of the method did not permit differentiation between faecal samples derived from different subjects at a statistically significant level.

In another study by Apperloo-Renkema and co-workers [14], titres of circulating autoantibodies (of the IgG, IgM and IgA-isotype), directed against 28 morphologically distinct subsets of faecal objects (mostly bacteria) were assessed by means of a combined quantitative immunofluorescence–morphometrical method. The distribution of the titres of circulating Ig over these morphologically distinct subsets or 'morphotypes' was found to be unique per volunteer despite the fact that their method yielded only semi-quantitative results with moderate accuracy.

In this paper an improved fluoromorphometrical method is presented which allows quantitative differentiation between faecal samples derived from different subjects. In short, the distribution of circulating antibodies over 144 faecal morphotypes is quantified by means of an image analysis system and quantitative immunofluorescence. Furthermore, a dataprocessing method was developed which allows objective quantitation of the data obtained by means of this improved method.

The results of this study imply that differentiation between faecal microflora derived from different subjects, on the basis of their binding to circulating antibodies, is possible.

MATERIALS AND METHODS

Volunteers

Eight male and two female healthy volunteers, aged 22–61 years, each provided one faecal sample and one serum sample. No one had received antimicrobial chemotherapy during the 2 months preceding the study, and no one had altered dietary habits in the same period. All volunteers gave informed consent before participating. The study was reviewed and permitted by the medical ethical committee of the University Hospital of Groningen.

Preparation of the immunofluorescence slides

Prior to use, slides (Immunocor, France) were degreased for 10 min in acetone. Half a gram of faeces was suspended in 4·5 ml of a solution of 0·5% Tween-80 in sterile water and thoroughly mixed. After centrifugation of this faecal suspension (10 min, 7 g) in a Beckman centrifuge type TJ-6 (Palo Alto, California, USA), the supernatant was collected and diluted 1 in 25 in a 0·5% Tween-80 solution and 10 μl of this diluted supernatant was pipetted in each of the 12 wells per slide. After drying on a hotplate at 37 °C, the faecal bacteria were fixed to the slides for 10 min in acetone. Thereafter, the faecal bacteria of each volunteer were incubated with his/her own serum. Briefly, the slides were incubated for 45 min at room temperature with 20 μl of a matching serum dilution (1:10 in phosphate buffered saline (PBS, pH 7·2 at 37 °C) added to each well. After washing the slides three times in PBS, each well was incubated with 20 μl of a solution (1:100 in PBS) of fluorescein-isothiocyanate(FITC)-conjugated goat, anti-human F(ab′)2 IgA, IgG and IgM (Kallestad, Texas, USA) for 1 h at room temperature in a moist chamber. Finally the slides were washed three times in PBS and mounted in a solution

comprising glycerol/TrisHCL buffer, v/v 1:1, pH 8·6. PBS was added as a negative control in place of the diluted serum. All samples were assayed in duplicate.

Fluoro-morphometrical analysis

The hardware and software of the image analysis system have been described previously [15]. Briefly, an Orthoplan UV-microscope (Leitz, Germany) with a phase-contrast condensor, a mercury lamp (HBO 100 W, Osram, Germany) and a CCD video camera (Fairchild CCD 5000/1, Sunnyvale, California) were used. The image analysis system comprises a Compaq deskpro 80486 microcomputer with 8 MB RAM equipped with a MATROX MVP/AT image-processing board (MATROX Electronic Systems Ltd, Quebec, Canada) and an exposure-control expansion board interfacing the computer to the CCD. By means of this board, which was developed in our laboratory [16], the integration time of the camera can be increased from one video frame ($= 1/30$ s) to any integer number of video frames. In this study an integration time of 128 video frames was employed. Analysis of the phasecontrast image of a microscopical slide results in a binary image containing only background and faecal objects. In addition, a fluorescence image is generated containing the fluorescence intensities of the objects in the accompanying binary image.

Objective description of the microscopic objects requires quantitative morphological parameters. Meijer and colleagues [17] derived four such parameters based on four measurements: surface area (A), perimeter (P), moment of inertia (I) and area of convex hull (H). The moment of inertia is defined as:

$$I = \sum_{i} [(x_i - \bar{x})^2 + (y_i - \bar{y})^2], \tag{1}$$

in which the x_i are the x coordinates of the pixels belonging to the object, and $\bar{x}$ is their mean; likewise for y_i and $\bar{y}$. Based on these four measurements, the morphological parameters derived were:

$$a = \log A, \tag{2}$$

$$f1 = 2\log P - \log A, \tag{3}$$

$$f2 = \log I - 2\log A, \tag{4}$$

$$c = \log H - \log A. \tag{5}$$

Next, four principal component scores (F1, F2, F3 and F4) were calculated from these morphological parameters. The matrix of transformation coefficients applicable on the entire data set was created previously by Meijer and colleagues [17] using 58 samples of faecal bacteria from nine healthy volunteers. It was decided to use two principal components (F1 and F2) which account for most (i.e. 93·5%) of the original variance.

Besides the morphometrical information obtained from a microscope image, the same image is recorded under ultraviolet illumination yielding the fluorescence intensity per faecal object. In pure cultures of *Enterobacter* spp., Apperloo-Renkema and co-workers have shown that the fluorescence intensity is linearly correlated with the amount of fluorescent circulating antibodies bound to the objects on the microscopic slide [18]. Fluorescence intensities are expressed as grey-

values ranging from zero (= no fluorescence) to 255 (= fluorescence intensity is equal to, or larger than the capacity of the camera/digitizer combination).

In serological procedures it is common use to express antibody concentrations as a titre. Titre is defined as the inverse of the dilution which is needed to satisfy some arbitrary condition. This arbitrary condition is also designed as the threshold value. In this paper the fluorescence level is used instead of the titre as a descriptor of the antibody concentration because the fluorescence level does not depend on a threshold value and is, therefore, an objective measure of antibody concentration.

Data analysis

The region of the morphometrical plane (defined by: F1 × F2) where at least 99% of the faecal objects are positioned is confined to: $-2 \leqslant F1 \leqslant 8$ and $-5 \leqslant F2 \leqslant 5$. This morphometrical region is then arbitrarily divided into 12 × 12 equal sized squares. The number of squares is only dictated by computer performance. Each square defines a morphotype because objects within the borders of a morphotype appear uniform to the observer. Per morphotype the median fluorescence of the objects it contains minus the median fluorescence of the objects in the corresponding morphotype of the negative control is computed, yielding a datavector of 144 fluorescence intensities.

In 144 dimensional space, the length of a datavector reflects the overall fluorescence intensity, while the direction of the vector reflects the distribution of median fluorescence intensities over 144 morphotypes. Because the overall fluorescence intensity of FITC-labelled bacteria strongly depends on the process of slide manufacture, the direction of the vector rather than the length is regarded as specific per individual. Differences in binding of circulating antibodies to faecal microflora between subject A and B are reflected by the angle between their corresponding datavectors. Using euclidian metric, the angle between vector **A** and vector **B** can be computed by means of a formula 6.

$$\cos \beta = \frac{\mathbf{A} \cdot \mathbf{B}}{|\mathbf{A}| \times |\mathbf{B}|}. \tag{6}$$

Where $\mathbf{A} \cdot \mathbf{B}$, $|\mathbf{A}|$ and $|\mathbf{B}|$ represent the inner product of datavectors **A** and **B**, the euclidian length of vector **A** and the euclidian length of vector **B**, respectively.

On the basis of the angle between the datavectors (which ranges from zero to 90 degrees) a similarity coefficient (S) is constructed which allows a conceptually easier way of data-interpretation. This similarity coefficient is defined as:

$$S = 1 - (\beta/90), \tag{7}$$

where S is the similarity coefficient between datavector **A** and **B** and β the angle between **A** and **B**. When the two datavectors are equidirectional their angle is zero degrees and S is 1 and when **A** and **B** are at an angle of 90 degrees S equals zero.

RESULTS

As an example, the fluorescence distribution over some of the faecal morphotypes (defined by an F1-range of -1.5 to 3.0 and an F2-range of -2.5 to
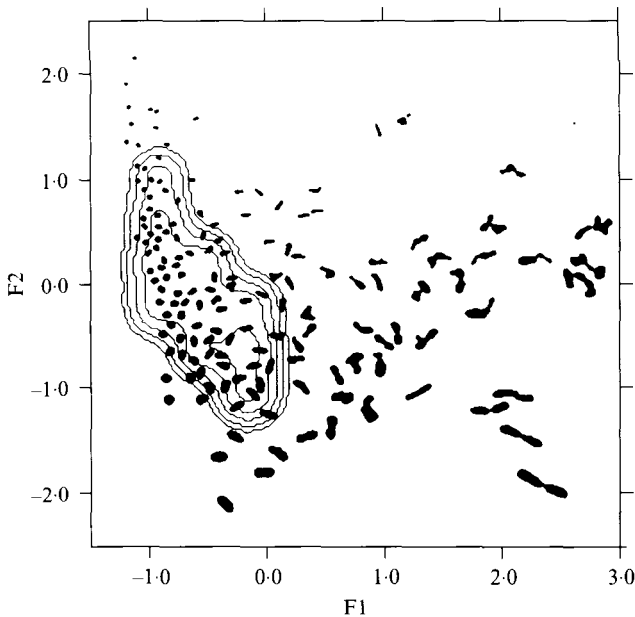
Fig. 1. Binding of systemic IgG to faecal bacteria. Combined scatterplot and contour plot. Objects in the morphometrical plane are represented as bacteria instead of symbols. For reasons of clarity some bacteria are omitted. The part of the (F1 × F2)-plane which is enclosed by $-2 < F1 < 8$ and $-5 < F2 < 5$ is divided into 12 × 12 fields or morphotypes for further correlation calculations. The contours represent median fluorescence intensities per morphotype. The outer contour represents objects with a fluorescence level of 2·00. Subsequent contours indicate the location of objects which exhibit a fluorescence intensity twice as large as the contour by which it is enclosed.

Table 1. *Similarity coefficients between datavectors of 10 faecal samples using an FITC-labelled goat anti-human anti-IgA conjugate*

	A	B	C	D	E	F	G	H	I	J
A	1·000									
B	0·426	1·000								
C	0·276	0·394	1·000							
D	0·347	0·248	0·333	1·000						
E	0·418	0·249	0·312	0·590*	1·000					
F	0·363	0·371	0·379	0·290	0·312	1·000				
G	0·009	0·038	0·012	0·102	0·083	0·062	1·000			
H	0·387	0·319	0·363	0·493*	0·468*	0·276	0·058	1·000		
I	0·269	0·402	0·418	0·181	0·319	0·341	0·109	0·297	1·000	
J	0·319	0·387	0·521*	0·333	0·333	0·319	0·051	0·371	0·234	1·000

* Similarity coefficient is larger than significance level (mean similarity between 10 duplicate measurements minus twice the standard deviation) which comprises 0·465 for IgA.

2·5) of subject A using FITC-labelled anti-IgG conjugate is depicted in Figure 1. Instead of symbols, the faecal objects themselves are drawn on the spots corresponding to their factor scores, though some of the objects have been omitted to enhance clarity. Fluorescence intensities are represented by means of a contourplot. Unenclosed objects exhibit a fluorescent intensity less than 2·00 which renders these objects indistinguishable from autofluorescent objects.

Table 2. *Similarity coefficients between datavectors of 10 faecal samples using an FITC-labelled goat anti-human anti-IgG conjugate**

	A	B	C	D	E	F	G	H	I	J
A	1·000									
B	0·408	1·000								
C	0·476	0·394	1·000							
D	0·476	0·312	0·261	1·000						
E	0·354	0·269	0·357	0·241	1·000					
F	0·319	0·241	0·331	0·221	0·270	1·000				
G	0·442	0·236	0·442	0·311	0·371	0·276	1·000			
H	0·400	0·418	0·442	0·201	0·318	0·322	0·356	1·000		
I	0·476	0·418	0·476	0·269	0·450	0·312	0·442	0·467	1·000	
J	0·459	0·221	0·276	0·269	0·221	0·275	0·378	0·319	0·319	1·000

* All similarity coefficients are smaller than the significance level obtained from ten duplicate measurements with anti-IgG.

Table 3. *Similarity coefficients between datavectors of 10 faecal samples using an FITC-labelled goat anti-human anti-IgM conjugate*

	A	B	C	D	E	F	G	H	I	J
A	1·000									
B	0·211	1·000								
C	0·337	0·227	1·000							
D	0·283	0·181	0·482	1·000						
E	0·386	0·158	0·493	0·494	1·000					
F	0·442	0·283	0·494	0·468	0·591	1·000				
G	0·297	0·148	0·363	0·371	0·312	0·348	1·000			
H	0·368	0·141	0·484	0·493	0·494	0·590	0·418	1·000		
I	0·248	0·116	0·402	0·468	0·371	0·379	0·371	0·637*	1·000	
J	0·402	0·304	0·333	0·283	0·297	0·434	0·304	0·312	0·248	1·000

* Similarity coefficient is larger than significance level (mean similarity between 10 duplicate measurements minus twice the standard deviation) which comprises 0·627 for IgM.

Interpretation of the similarity data requires a significance criterion. It was decided to use the mean similarity coefficient between duplicate measurements for all individual minus twice the standard deviation. This procedure is valid because the similarity between two datavectors derived from one subject is determined by inconsistencies in the process of slide manufacturing solely and not by individual-related characteristics. After the normality of the distribution of the similarity coefficients had been determined by means of the Lilliefors test [19], mean and standard deviations were computed for all three isotypes. The significance levels computed this way amount to 0·465, 0·578 and 0·627 for IgA, IgG and IgM, respectively. In Tables 1–3 the similarity coefficients between pairs of samples. using IgG, IgA and IgM are presented.

DISCUSSION

The human faecal microflora is a very complex ecosystem. It is therefore obvious that the classification of faecal bacteria into 144 morphotypes has no relation with conventional bacteriology. Morphotypes involve faecal objects which have no relationship with bacterial genera or families. They may even

include some non-bacterial objects. It was shown by Meijer that the bacteria in a pure culture of *Enterobacter intermedius* were not confined to one morphotype but ranged over several adjacent morphotypes [20]. The results of this study, therefore, have to be interpreted in terms of humoral reactivity against morphologically similar faecal bacteria.

From the similarity matrices it can be deduced that the distribution of circulating specific IgG over the faecal morphotype renders differentiation between all samples in the trial possible at a statistically significant level. This probably reflects the high specificity of antibodies of the IgG isotype. Circulating antibodies of the IgM isotype, which are often less specific because of their biological function as a first line of humoral defence, allow sample separation, except for samples H and I. Though it needs to be mentioned that the similarity coefficient between H and I is only slightly (i.e. 1·6 %) larger than the significance level computed for the IgM datavector measurements.

Circulating IgA is also less suitable for characterization purposes. This is to be expected on the basis of total circulating IgA measurements [14] which exert larger variability than total IgG and total IgM. An explanation of this phenomenon could be the observation that *in vivo* secretory IgA (sIgA) is produced by plasma cells located in the intestinal tract. Relatively high fluorescence intensities per morphotype of the PBS incubated negative controls of IgA indicate that the *in vivo* sIgA coating of faecal bacteria is measurable with this method. This coat of sIgA probably interferes with the binding of circulating IgA resulting in an extra source of methodological noise.

In conclusion, using quantitative immunofluorescence, image analysis and the dataprocessing procedure presented in this study, the distribution of circulating antibodies over 144 distinct faecal morphotypes yields a reproducible pattern of fluorescence-levels which is (depending on the Ig-isotype) more or less unique for each volunteer. For characterization of the faecal microflora and for studies concerned with the interaction between microflora and the humoral immune system of the host, this method is fast, accurate and highly sensitive. Future studies dealing with the longitudinal variability of the composition of the faecal microflora and the influence of food additives, by means of this method are currently undertaken.

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