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## **Effects of ceftriaxone on faecal flora: analysis by micromorphometry**

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### SUMMARY

In order to elucidate the effect of ceftriaxone therapy on the morphology of gut microflora, 11 human volunteers were treated with ceftriaxone, 1 g daily, given intramuscularly in one dose. Treatment continued for 5 days. Faecal microflora was analysed by digital image processing before, during and after the treatment period.

We derived simple numerical parameters which describe the morphologic composition of the flora. They were significantly influenced by the antibiotic, and returned to their baseline values more than 7 days after treatment was stopped. The procedure holds promise for clinical application.

### INTRODUCTION

Ceftriaxone is a relatively new cephalosporin, known for its broad antimicrobial spectrum and long serum half life. It is mainly excreted by the liver, and therefore may reach high levels in the gut lumen. The resident microflora of the mammalian gut is held responsible for colonization resistance. Therefore it is clinically relevant to have fast monitoring methods for its ecological balance. This balance is sensitive to antibiotic treatment [1]. Various methods have been employed to study this influence: aerobic and anaerobic culturing [2–8], determination of the fatty acid pattern in faeces [3, 9], and microflora associated characteristics [10, 11].

Digital image analysis (see e.g. the texts by Gonzalez and Wintz [12] and by Serra [13]) has recently started to be applied to microbiology [14–17]. It was shown by Meijer and co-workers that pure cultures could be distinguished, and mixtures containing three different species could be decomposed, using image analysis [14]. In the present study the changes in morphometrical parameters of human gut flora due to treatment with ceftriaxone are described. It was part of a series of studies on the influence of ceftriaxone on gut flora, performed in 1989 in our

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laboratory. Aim of the study was to develop morphometrical indicators of gut flora disturbance. These indicators should be reliable and easy to obtain.

#### MATERIALS AND METHODS

*Subjects.* Eleven healthy human volunteers (mean age: 34; range: 22–42; eight male, three female) were treated once daily with 1 g of ceftriaxone, injected intramuscularly in combination with lidocaine, for 5 days. Informed consent was obtained from all volunteers; the study was approved by the Commission for Medical Ethics of the Academic Hospital of Groningen. Faeces were collected from 7 days before to 22 days after the beginning of treatment. It was not possible to obtain faeces every day from each volunteer: 218 specimens were analysed in total. There were at least two pre-treatment specimens from each volunteer. Days were numbered as follows: the first dose was given on day 0, previous days being numbered -1, -2, etc. On day 0, faeces were collected before the first dose was given. The last dose was given on day 4.

*Slide preparation.* Microscopic slides were prepared from the faecal samples, using the nigrosine smear technique described earlier [14, 18]: 0.5 g of faeces was suspended in 4.5 ml of 0.5% Tween 80 in water, and thoroughly mixed. Samples were centrifuged at 15 g for 10 min. 150  $\mu$ l of supernatant fluid was centrifuged for 15 min at 10000 g; finally the pellet was resuspended in 150  $\mu$ l of nigrosine, 5% in 0.5% Tween 80. 5  $\mu$ l of this mixture was spread on a microscopic slide, using a second slide at a 45° angle. Twenty fields of view were examined on each slide.

*Ceftriaxone concentrations in faeces.* De Vries Hospers and colleagues (personal communication) determined faecal ceftriaxone concentrations every day. Data from these measurements were used with the authors' permission.

*Optical equipment.* An Olympus BHS microscope was used to view the slides, with a 100 $\times$  objective lens (numerical aperture 1.30) and a Sony CH 1400 video camera. Total magnification was such that the distance between adjacent pixels in the digital image corresponded to 0.126  $\mu$ m in the object.

*Image analysis.* We used a PC/AT compatible computer, with a PIP 1024 video digitizer board (Matrox, Quebec, Canada). The board has four 512 $\times$ 512 $\times$ 8 bit frame stores and a programme library of basic image processing functions is provided with it. Further software, including a command language interpreter, a library of more complex image processing functions, and programmes for manipulating binary images, was written by the authors. For a description the reader is referred to an earlier publication [14]. The software has since been adapted to the MVP/AT, a newer video digitizer by the same manufacturer.

In this study, image analysis proceeded as in our earlier work: after obtaining the image, an out-of-focus image was subtracted from it to eliminate slow variations in background intensity. The Y axis was mapped to three-quarters of its original length to correct for pixel aspect ratio. Averaging with a 3 $\times$ 3 kernel was performed. Then, the robust automatic threshold selector algorithm described by Kittler and co-workers [19] was used to separate objects and background. The objects in the resulting binary image were visually screened by the operator and artifacts were deleted. Remaining objects, including diplococci, streptococci, etc,

were analysed as if they were single bacteria. From each portion of faeces, at least 1000 bacteria were analysed whenever possible. Four measures were taken of each bacterium: surface area (A), perimeter (P), moment of inertia (I), and convex hull area (H). The moment of inertia can be defined in terms of the centralized moments described by Gonzalez & Wintz [12; p 356]. In a formula:

$$I = \mu_{20} + \mu_{02}$$

with

$$\mu_{20} = \Sigma(x_i - \bar{x})^2$$

and

$$\mu_{02} = \Sigma(y_i - \bar{y})^2$$

where the  $x_i$  are the  $x$  coordinates of all pixels belonging to the object, and  $\bar{x}$  is their mean; likewise for  $y_i$  and  $\bar{y}$ . The moment of inertia is rotation-invariant. When objects with equal areas are compared, elongated objects will have large values of I.

*Logarithmic transformation.* The raw data were then transformed to eliminate most of their mutual dependence and to improve the linearity of remaining relations between the variables. Natural logarithms were computed, and combined into one size indicator (a) and three size-independent measures of shape ( $f_1$ ,  $f_2$  and c):

$$a = \log A, \text{ the natural logarithm of area,} \quad (1)$$

$$f_1 = 2 \log P - \log A, \text{ the first form factor,} \quad (2)$$

$$f_2 = \log I - 2 \log A, \text{ the second form factor,} \quad (3)$$

$$c = \log H - \log A, \text{ the concavity factor.} \quad (4)$$

*Principal components transformation.* Next, three principal component scores were computed from a,  $f_1$ ,  $f_2$  and c. To obtain a matrix of transformation coefficients usable with all data, a mixed data file was created from measurements on the first pre-treatment day of all volunteers. On this file we performed principal component analysis, and the resulting score coefficients were used throughout the rest of the experiment to compute component scores. A more detailed treatment of data transformations applied to pure cultures has been published elsewhere [14].

*Estimating biological variety.* We presumed that the presence of ceftriaxone would select among the species originally present in the gut and thus reduce biological and morphometrical variety. To estimate this variety the following procedure was used: the intervals from  $-5$  to  $5$  for each component were divided into 16 bins, so that the cube of morphometrical space extending from  $-5$  to  $5$  in all three directions was cut into 4096 smaller cubes. The probability for a measurement point to fall into any one of these cubes was estimated by dividing the number that did fall into the cube by the number of observations:

$$\hat{p}_i = \frac{n_i}{N}.$$

Then, an entropy measure was constructed by

$$S = - \sum_{i=1}^{4096} \hat{p}_i \log \hat{p}_i$$

again using the natural logarithm.

*Statistical analysis.* For each specimen, medians were computed of the three component scores. This yielded three numbers for each available combination of subject and day. They were used separately in statistical testing. Overall differences between days were tested nonparametrically, according to Friedman [20]. Before applying the Friedman test, missing data were filled in: if no measurements were available for a subject on a certain day, we substituted the arithmetic mean of all days for that subject. This method reduces the sensitivity of the test, but it is conservative. Next, pairs of subsequent days were tested for significant difference, using a Wilcoxon symmetry test [20]. No special treatment of missing data was necessary here: subjects with one or both days of the pair missing were not included in the test.

*Graphic representation.* Modified scatter plots were made of selected specimens. In these plots, principal components 1 and 2 are used as  $X$  and  $Y$  coordinates. Instead of symbols, the bacteria themselves are drawn on the spots corresponding to their factor scores. The four morphometric variables: the three medians and entropy, were plotted against time for two subjects chosen to represent extremes of possible response to treatment. In order to facilitate comparison, each variable was standardized as follows: for each subject a pre-treatment average was computed and subtracted from the values for that subject. Next, a pre-treatment pooled standard deviation was computed and the values for all subjects were divided by that standard deviation.

## RESULTS

For nearly all specimens, the number of bacteria analysed was between 500 and 1000. Due to killing of bacteria by the ceftriaxone, in some cases this number was not achieved in 20 fields of view. These cases were subject 9, who had 162 bacteria on day 6; subject 10: 384, 56, 74 and 679 bacteria on days 3, 4, 5 and 6, and subject 11: 281 bacteria on day 2.

For all three component medians and entropy, the overall differences between days proved significant (Friedman test;  $P < 0.1\%$ ). The Wilcoxon tests between subsequent days showed significant differences for all medians and entropy between days 1 and 2 ( $P < 5\%$ ). Due to missing data, only 174 of 216 possible pairs (80.6%) were complete. Between days 1 and 2, the comparison was applied to 9 of the 11 subjects.

There were 40 faecal specimens from the pre-treatment period. Friedman and Wilcoxon tests on this period yielded no statistical significance.

Figure 1 shows a modifier scatter plot from the pre-treatment period (subject 3, day -6). In Figure 2, taken on day 5 from the same subject, the change is notable. Figures 3 and 4 show the medians of the three principal components, the entropy, and the faecal ceftriaxone concentration as functions of time for subjects 2 and 3, respectively. The ceftriaxone concentration is plotted against a logarithmic axis.

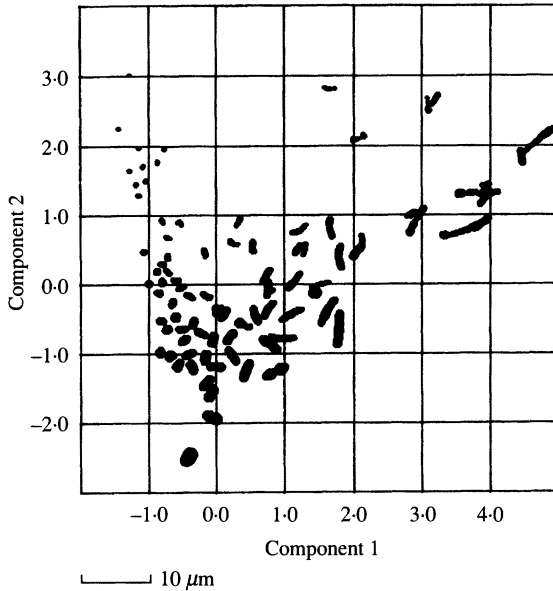


Fig. 1. Modified scatter plot (see text) of subject 3 on day -6 (before treatment).

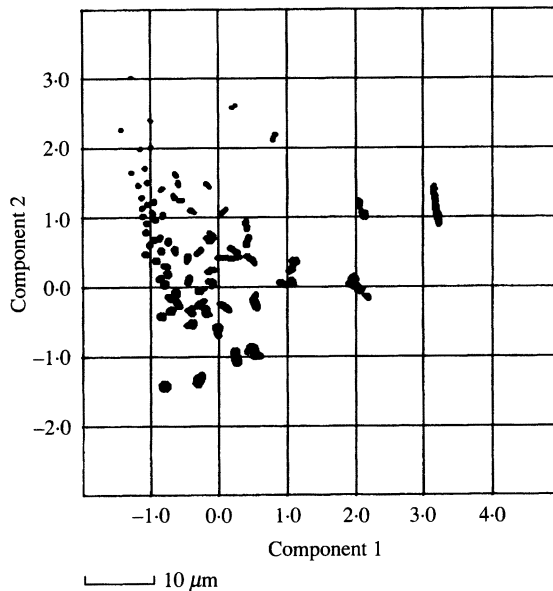


Fig. 2. Modified scatter plot (see text) of subject 3 on day 5 (immediately after treatment).

The medians and the entropy were standardized as described above and plotted against a linear axis. A significance level of 5% is attained when the absolute value of the standardized variable exceeds 2.04 (Student distribution, 29 degrees of freedom, two-tailed; for practical purposes: 2.0).

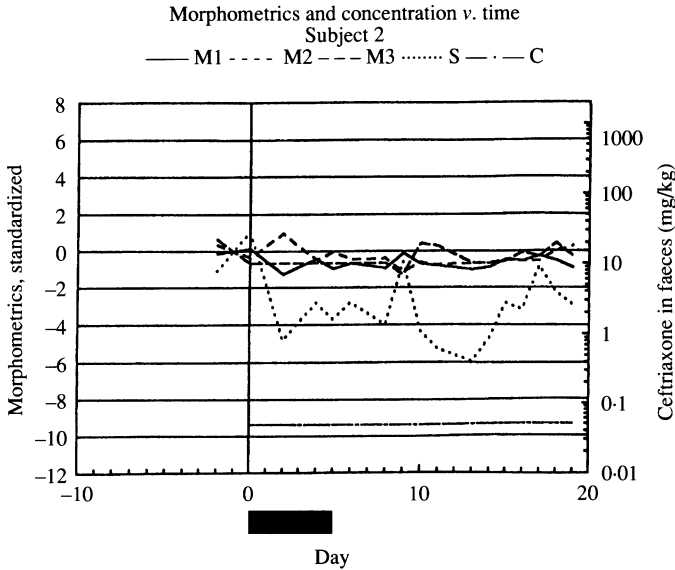


Fig. 3. Standardized medians 1, 2 and 3 (M1, M2, M3, resp.), standardized morphometric entropy (S), and ceftriaxone concentration (C) as functions of time for subject 2. The black bar indicates the treatment period.

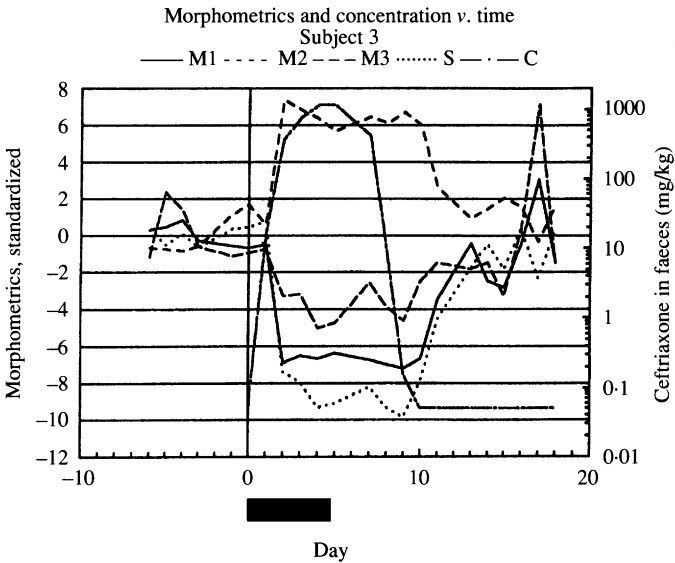


Fig. 4. Standardized medians 1, 2 and 3 (M1, M2, M3, resp.), standardized morphometric entropy (S), and ceftriaxone concentration (C) as functions of time for subject 3. The black bar indicates the treatment period.

In Table 1 the behaviours of the median of component 1, the entropy, and the ceftriaxone concentration as functions of time are summarized. Median 1 shows significant change in 5 of 11 subjects (45%), median 2 in 8 of 11 (73%), median 3 in 4 of 11 (36%) and the entropy in all 11 subjects.

Table 1. Summary of patterns found in morphometrical parameters and faecal ceftriaxone concentration

Subject	Ceftriaxone detected in faeces	Median 1	Median 2	Median 3	Entropy
1	No	d	i	d	d
2	No	-	-	-	d
3	Yes	d	i	d	d
4	Yes	d	i	d	d
5	Yes	-	i	-	d
6	Yes	d	i	-	d
7	No	d	i	d	d
8	No	-	-	-	d
9	No	-	i	-	d
10	Yes	-	i	-	d
11	Yes	-	-	-	d

Symbols: i: increased significantly during or after treatment; d: decreased significantly during or after treatment; -: no change.

#### DISCUSSION

The results show that digital micromorphometry is sensitive to the effects of ceftriaxone on faecal microflora. Moreover, the influence on morphometric parameters is consistent when various subjects are compared. Generally, the following effects are seen during treatment. Principal component 1 is decreased, component 2 is increased and component 3 is decreased a little. Morphologically, the changes correspond to a relative preponderance of coccoid, small, convex bacteria, respectively. The morphometrical diversity as measured by our estimates for entropy is decreased. For those subjects in whom the faecal level of ceftriaxone is above the detection limit, the duration and severity of these effects correlate well with duration and height of ceftriaxone level in the faeces of the subjects. Interestingly, a consistent effect on the morphometric entropy is also found in those subjects in whom faecal ceftriaxone remained below the detection limit. In this experiment, entropy was the most sensitive morphometric indicator of disturbance in gut microflora.

Digital micromorphometry is fast compared to culturing anaerobic bacteria. It can be used to examine specimens from patients regularly and so monitor their gut flora. Before the technique can be used clinically, the method will have to be thoroughly validated. Reproducibility and accuracy of measurement can be determined by comparing duplicate measurements on the same field of view. This has been done; results will be published shortly. Then, it is plausible to assume a causal relationship between the results of anaerobic culture and those of morphometry. This relationship has to be elucidated by comparison of morphometrical and microbiological data, obtained from the same specimens. As anaerobic culturing was done concurrently from the specimens obtained in this experiment, there is an excellent basis for comparison. Results will be published in the near future.

Although the fact that no consistent differences were found in the pre-treatment



period (Friedman and Wilcoxon tests) points out that random variation in morphometrical parameters is small in comparison to variation due to ceftriaxone treatment, more insight is needed in the long term stability of those parameters in healthy untreated humans. This problem has been addressed by a study of micromorphometry of volunteers who were followed for 8 weeks without treatment.

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